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(57) Abstract

Polypeptides derived from hepatitis B surface antigen which are able to compete with the hepatitis B surface antigen/annexin V interaction, or which are able to bind a compound or antibody competing with the hepatitis B surface antigen/annexin V interaction are described. The use of these polypeptides, and antibodies against them, in order to diagnose, treat and vaccinate against an infection with hepatitis B virus and/or hepatitis delta virus is also disclosed.

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ANNEXIN V-BINDING POLYPEPTIDES DERIVED FROM HBsAg AND THEIR USES

FIELD OF THE INVENTION

The present invention relates to polypeptides derived from a hepatitis B virus envelope glycoprotein, termed hepatitis B surface antigen, which compete with the interaction between hepatitis B surface antigen and the phospholipid-binding protein annexin V. The present invention also concerns the use of these polypeptides, and antibodies against them, in order to diagnose, treat and vaccinate against, an infection with hepatitis B virus and hepatitis delta virus.

BACKGROUND OF THE INVENTION

Hepatitis B virus (HBV) belongs to the Hepadnaviridae which are characterized by a significant hepatotropism and species specificity. Hepatitis delta virus (HDV) represents a naturally occurring subviral satellite of HBV (Rizetto et al, 1986). HBV causes major medical problems, such as chronic liver disease and hepatocellular carcinoma (Schroder & Zentgraf, 1990). HDV superinfection is usually more severe compared to HBV infection solely. It is estimated that there are 300 million human carriers of the virus worldwide, while in the US only 70,000 carriers are coinfected with HDV.

Within the HBV genome, and more particularly within the S gene (see also next paragraph), there exists a natural sequence variation. Genotypes A to F of HBV are designated based on this sequence divergence (for review, see Magnius & Norder, 1995).

The HBV envelope consists of three related glycoproteins, termed hepatitis B surface antigens (HBsAg), which are the product of the S gene: 1) the "small" transmembrane protein, also termed major protein or small S-protein, composed of 226 amino acids (aa), 2) the "middle" protein which comprises the small S-protein and 55 additional aa at the N-terminus corresponding to the pre-S2 region of the S gene, and 3) the "large" protein composed of 389 or 400 aa corresponding to the following regions: S + pre-S2 + pre-S1 (108-119 N-terminal aa) (Heerman et al., 1984; Robinson et al., 1987). The envelope of HDV is also entirely derived from HBV and consists predominantly of small HbsAg, 5-10% of middle HbsAg and no or less then 1% of large

HbsAg (Bonino et al., 1986).

The region composed of aa 100 to 160 of "small" HBsAg has been predicted to be located on the outer surface of the virus (for review, see Berting et al., 1995). Current vaccines, using recombinant "small" HBsAg, result in a protective antibody response directed against a limited number of epitopes such as the major "a" determinant: aa 139-147. The latter determinant is shown to be the most immunodominant epitope within the region aa 100-160 of "small" HBsAg (for review, see Magnius & Norder, 1995, and, Howard & Allison, 1995). On the other hand, many vaccine escape mutants for the "a" determinant have evolved since (Howard & Allison, 1995). There is thus a need to characterize new epitopes which can be used in the design of new vaccines against hepatitis B.

Annexin V (also termed endonexin II, placental anticoagulant protein, PP4 or lipocortin V) is a member of the family of structurally related Ca^{2+}-dependent phospholipid-binding proteins, known as annexins, which have molecular weights between 32 and 67 kDa (Klee, 1988; Zaks & Creutz, 1990). Annexin V is found in various tissues such as liver, spleen, lung, intestine and placenta (Walker et al., 1990). The protein has been described to bind, in a Ca^{2+}-dependent manner, to placental membranes (Haigler et al., 1987) and to inhibit blood coagulation (Grundman et al., 1988) and phospholipase A2 activity *in vitro* (Pepinsky et al., 1988). Other investigators have demonstrated that annexin V behaves like an integral membrane protein and forms calcium-selective cation channels (Rojas et al., 1990; Bianchi et al., 1992).

We have recently shown that annexin V, present on human liver plasma membranes, specifically binds to "small" HBsAg in a Ca^{2+}-dependent manner (Hertogs et al., 1993; WO 94/01554). The receptor-ligand relationship between HBsAg and annexin V is further supported by the observation that rabbits, immunized with native human liver annexin V or recombinant annexin V, or chickens, immunized with $F(ab')_2$-fragments of rabbit anti-annexin V IgG, spontaneously develop anti-idiotypic antibodies (Ab2) which specifically recognize HBsAg (Hertogs et al., 1994). We also demonstrated that HDV particles are binding to annexin V via the HbsAg containing envelope of HBV (de Bruin et al., 1994). However, and although several peptides derived from HbsAg have been described (EP 044710; EP 0155146; WO 9516704) the exact site on HBsAg which binds annexin V was not known. Mapping of this site could lead to the development of new epitopes which can be used in the design of methods to interrupt the infection cycle of both HBV and HDV.

AIMS OF THE INVENTION

It is clear from the literature cited above that the appearance of vaccine escape mutants to the major "a" determinant of "small" HBsAg presents a problem with regard to the protection offered by the currently used vaccines against HBV infection. Indeed, HBV infections have been described in vaccinated children (Howard et al., 1995). The present invention aims, in general, at providing new polypeptides which, when used in a vaccine or any other composition, result in a better protection against hepatitis B, and consequently hepatitis D, infection.

A polypeptide, used in a vaccine composition, which might provide a better protection is very likely derived from a viral antigen which plays a crucial role for survival of the virus. As it has been demonstrated that annexin V binds HBsAg and, thus, might play a vital role in the initiation of HBV and HDV infection, the annexin V-binding domain on HBsAg may not be subject to major mutations. This is in contrast to the "a" determinant on HBsAg for which many escape variants have already been characterized (Carman et al., 1990). In other words, the development of escape mutants in the annexin V-binding domain of HBsAg is unlikely. Therefore, the present invention aims at providing a polypeptide derived from HBsAg, which competes with the HBsAg/annexin V interaction or which binds a compound or antibody competing with the HBsAg/annexin V interaction, and is immunogenic. More specifically, the present invention aims at providing a polypeptide as described above which comprises less than 61 amino acids containing at least 4 amino acids of one of the following sequences: FAKYLWEWASVR, KTCTTPAQGN and TTPAQGN. In this regard, it should be clear that mapping such a polypeptide is not an obvious undertaking. In particular, engineering a peptide in such a way that it evokes upon immunization a specific response against the annexin V-binding epitope or a part thereof, is not obvious at all.

The present invention further aims at providing a polypeptide which comprises the sequence KTCTTPAQGN or TTPAQGN, and the sequence FAKYLWEWASVR, or functionally equivalent parts or variants of siad sequences.

The present invention also aims at providing a vaccine composition which comprises as an active substance a polypeptide as described above.

Furthermore, the present invention aims at providing a vaccine composition as described above for use as an inoculum to vaccinate humans against infection with HBV and/or HDV or any

mutated strain thereof or to therapeutically vaccinate human carriers of HBV and/or HDV or any mutated strain thereof. The reason to be able to therapeutically vaccinate carriers of HBV using the above-described vaccine composition is based on our surprising observation that the polypeptide derived from HBV, as described above, evokes a specific antibody response against this polypeptide whereas a natural HBV infection in a control chimpanzee (see Example 5) does not result in the production of such antibodies. In other words, the inoculation of the above-described vaccine composition during an infection with HBV results in a better immune response and hence a better protection.

Thus, the present invention also aims at providing a polypeptide as described above or any mutated version thereof which, upon inoculation in a mammalian host, results in the production of antibodies which specifically bind to said polypeptide, in particular to the sequence KTCTTPAQGN or a part thereof, or, to the sequence TTPAQGN or a part thereof, or, to the sequence FAKYLWEWASVR or a part thereof.

The present invention also aims at providing a combination of a polypeptide according to any of the previous aims and a negatively charged phospholipid.

Furthermore, the present invention aims at providing a polypeptide composition comprising any combination of polypeptides as defined above.

The present invention also aims at providing antibodies or fragments thereof which specifically bind to a polypeptide as defined above and inhibit binding of said polypeptide to annexin V.

The present invention further aims at providing a pharmaceutical composition comprising as an active substance the antibodies or fragments thereof as defined above for use in a method to treat humans infected with HBV and/or HDV or any mutated strain thereof.

Furthermore, the present invention aims at providing a method, using a polypeptide as defined above, to detect antibodies which are capable of competing with the HBsAg/annexin V interaction and which are present in a biological sample, comprising:

a) contacting the biological sample to be analysed for the presence of HBsAg antibodies with a polypeptide as defined above,

b) detecting the immunological complex formed between said antibodies and said polypeptide.

The present invention also aims at providing a pharmaceutical composition comprising as an active substance a polypeptide as defined above for use in a method to treat humans infected

with HBV and/or HDV or any mutated strain thereof.

Moreover, the present invention aims at providing a polypeptide as defined above for use in a method to screen for drugs which block the binding between annexin V and said polypeptide.

The present invention also aims at providing polypeptides as defined above for use as a medicament to treat humans infected with hepatitis B virus and/or hepatitis delta virus or any mutated strain thereof.

The present invention finally aims at providing a kit for the in vitro determination of antibodies to HBsAg present in human serum containing: at least one microplate, polypeptides as defined above, appropriate buffer, blocking and washing solutions which favor binding of said polypeptides with the antibodies in human serum samples, and appropriate markers which allow to determine the complexes formed between the antibodies in human serum samples and said polypeptides.

All the aims of the present invention are considered to have been met by the embodiments as set out below.

BRIEF DESCRIPTION OF DRAWINGS AND TABLES

Table 1 provides sequence information concerning the region aa 99 to 169 of "small" HBsAg which determines four genotypes of HBsAg (A,B,C and D) and regarding the polypeptides which were examined for binding to annexin V and to several antibodies and for their immunogenicity in chimpanzee and rabbits.

Table 2 shows the relative affinity of peptides for binding the the anti-HBsAg monoclonal, C11F5. Briefly binding of C11F5 to HBsAg coated on microtiterplates is competed with the peptides IGP 1076-1083 in soloution. The molar concentration of HBsAg itself yielding a 50% competition is equal to 1.

Figure 1 shows the influence of anti-idiotypic antibodies (Ab2) on the production of HBsAg in culture medium of primary cultures of adult human hepatocytes infected with HBV. HBsAg production of infected cells at days 3, 5, 7, 8 and 10 post-infection in the absence of anti-idiotypic antibodies is represented by "O". Results of HBsAg production in media of non-infected cells or

of cells infected in the presence of anti-idiotypic antibodies are represented by "+" and "▲", respectively. The findings presented are the average of results obtained from five experiments.

Figure 2 demonstrates that anti-idiotypic antibodies (Ab2) which specifically recognize the annexin V binding domain of HBsAg prevent HBV infection in primary cultures of human hepatocytes *in vitro*. Briefly, the figure represents an autoradiogram of the Southern blot analysis of HBV-DNA and replicative intermediates in primary cultures of human hepatocytes infected with HBV in the presence and absence of anti-idiotypic antibodies. Integrated HBV-DNA sequences were present in HepG2.2.15 (a HepG2 cell line transfected with the HBV genome (Sells et al., 1987) (lane 1). HBV-DNA replicative intermediates were detected in cells infected with HBV inoculum in the absence of anti-idiotypic antibodies (lane 4). Lane 2 and 3 represent the results of HBV-DNA detection in experiments without HBV inoculum and in parallel experiments in the presence of anti-idiotypic antibodies, respectively.

Figure 3 demonstrates the binding of an anti-idiotypic antibodies (Ab2), which mimic annexin V, to the polypeptides indicated in table 1 and "small" HBsAg. Briefly, the polypeptides or HBsAg were adsorbed to microtiterplates which were incubated after blocking with a serial dilution of Ab2 (1/50 to 1/5000). Binding of Ab2 was visualized using an anti-rabbit IgG preparation conjugated with peroxidase. Plates were developed using tetramethylbenzidine as colour reagent, finally plates were read at 450 nm.

Figure 4 shows the binding of Ab2 to biotinilated forms of some of the polypeptides indicated in table 1. Briefly, streptavidin was adsorbed to microtiterplates which were incubated after blocking with biotinilated peptides, followed by an incubation with serial dilutions of Ab2 (1/50 to 1/5000). Binding of Ab2 was visualized using an anti-rabbit IgG preparation conjugated with peroxidase. Plates were developed using tetramethylbenzidine as colour reagent, finally plates were read at 450 nm.

Figure 5 shows that HBsAg of either genotypes A or D binds Ab2 and annexin V. Briefly, a serial dilution of HBsAg was coated on microtiterplates. After blocking the plates were incubated with Ab2 (Figure 5A) derived from a rabbit immunized with annexin V or with annexin V itself labelled

with peroxidase (Figure 5B). Binding of Ab2 was visualized using an anti-rabbit IgG peroxidase conjugate.

Figure 6. Binding of horse radish peroxidase (HRPO) labelled recombinant human A-V to solid phase HBsAg in the presence of a serial dilution of phosphatidylserine (open symbols) or phosphatidylcholine (closed symbols).

Figure 7. Binding of HRPO labelled A-V to HBsAg in the presence of a serial dilution of mAbs directed against human A-V (U4C8: open triangles, U1E10: closed triangles, E1E8: open circles, CM1995: closed circles).

Figure 8. Relative inhibition, expressed as % of competition, to a positive control (experiment without competitor) of the binding of HRPO labelled A-V to solid phase mAb in the presence of a 75 molar excess of unlabelled rat or human A-V (CM1995: open bars, U4C8: hatched bars, U1E10: closed bars).

Figure 9. Relative inhibition, expressed as % of competition, to a positive control (experiment without competitor) of the binding HRPO labelled recombinant human A-V to solid phase mAb in the presence of excess of phosphatidylserine (open bars) or phosphatidylcholine (hatched bars) (ratio phospholipid/mAb is 30 (w/w)).

Figure 10. Visual impression of binding between HBsAg and A-V and of the binding sites of all inhibiting antibodies.

Figure 11 shows the antibody response of a chimpanzee vaccinated with some of the polypeptides indicated in table 1. Briefly, streptavidin or HBsAg was adsorbed to microtiterplates, streptavidin coated wells were incubated after blocking with biotinilated peptides (IGP 1103, 1119 and the control peptides 1030 and 1038), followed by an incubation with a serial dilution of the vaccinated chimpanzee serum (1/20 to 1/2560). Binding was visualized using an anti-human IgG preparation conjugated with peroxidase. In this regard, it should be noted that IgG antibodies of humans and chimpanzees show sufficient cross-reactivity so that chimpanzee IgG's can be detected using anti-

human IgG conjugates. Plates were developed using tetramethylbenzidine as colour reagent, finally plates were read at 450 nm.

Figure 12 demonstrates the evolution of antibody titer of a chimpanzee vaccinated with the polypeptides IGP 1103 and 1119 as described in table 1. Briefly, streptavidin or HBsAg was adsorbed to microtiterplates, streptavidin coated wells were incubated after blocking with biotinilated peptides (IGP 1103, 1119), followed by an incubation with a serial dilution of the vaccinated chimpanzee serum (1/20 to 1/2560). Binding was visualized using an anti-human IgG preparation conjugated with peroxidase. Plates were developed using tetramethylbenzidine as colour reagent, finally plates were read at 450 nm. Titer is expressed as the serum dilution still giving a positive signal compared to a negative control.

Figure 13 shows the antibody response of a chimpanzee challenged with HBV. Briefly, streptavidin or HBsAg was adsorbed to microtiterplates, streptavidin coated wells were incubated after blocking with biotinilated peptides (IGP 1103, 1119 and the control peptides 1030 and 1038), followed by an incubation with a single dilution of the vaccinated chimpanzee serum (1/20). Binding was visualized using an anti-human IgG preparation conjugated with peroxidase. Plates were developed using tetramethylbenzidine as colour reagent, finally plates were read at 450 nm. No specific reactivity with IGP 1119 or 1103 could be measured. The reactivity with the control peptides 1030 and 1038 always exceeded the reactivity with 1119 and 1103.

Figure 14 shows the cross reactivity of antibodies evoked by a genotype A derived peptide. Briefly, streptavidin was adsorbed to microtiterplates which were incubated after blocking with biotinilated peptides, followed by an incubation with a serial dilution of chimpanzee serum ($1/2^4$ to $1/2^{13}$). Binding of chimpanzee antibodies was visualized using an anti-human IgG preparation conjugated with peroxidase. Plates were developed using tetramethylbenzidine as colour reagent, finally plates were read at 450 nm. IGP 1030 represents a control peptide not related to HbsAg.

Figure 15 shows binding of annexin V to HBsAg peptides representing the amino acid region 112-169. Briefly, binding of annexin V (coupled with horse radish peroxidase) to HBsAg itself or to the biotinylated peptides IGP 671, 673 and 80 (representing the aa region 112-169), the

control peptide IGP 1038 and the peptide binding with the anti-idiotypic antibody (IGP 1119) coated to microtiterplates as a streptavidin complex was evaluated. The specificity of binding of the peptides to annexin V was demonstrated by adding an excess (100 µg/ml) of an anti-annexin V monoclonal antibody which is known to compete with the binding of annexin V to HBsAg (see further example 7).

Figure 16 shows further fine mapping of the annexin V binding site on HBsAg. Briefly, binding of a new series of peptides (IGP 1189-1193, IGP 1190-1192 being hetero-branched peptides, see Table 1) to annexin V was analysed as described above for Figure 15.

Figure 17 shows further fine mapping of the annexin V binding site on HBsAg by direct competition. Briefly, binding of annexin V to HBsAg coated on microtiterplates is competed with HBsAg and with the peptides IGP 1119 and IGP 1193 in solution (both peptides are presented as streptavidin complexes).

Figure 18 shows further fine mapping of the Ab2 epitope. Briefly binding of Ab2 to coated on microtiterplates is competed with the peptides P467-P471 in solution.

Figure 19 shows the mapping of the epitope for the monoclonal anti-HBsAg antibody, C11F5. Briefly peptides were adsorbed to microtiterplates as streptavidin complexes. Binding of C11F5 was visualized using a goat-anti-mouse antibody conjugated to peroxidase.

Figure 20 shows the binding of annexin V to peptides containing the HBsAg region 158-169. Briefly binding was performed as described for figure 15, but binding was performed at room temperature instead of 37°C, this in order to select for stronger binding peptides.

Figure 21 shows the relative affinity of peptides for binding to annexin V. Briefly, binding of annexin V to HBsAg coated on microtiterplates is competed with a 200 or 20 molar excess of peptide in solution.

Figure 22 shows the mapping of the antibody response of chimpanzee and rabbit against he epitope region 115-134. Briefly peptides were adsorbed to microtiterplates as streptavidin complexes. Binding of antibodies was visualized using a rabbit-anti-human or goat-anti-rabbit antibody conjugated to peroxidase.

Figure 23 shows the mapping of the antibody response of rabbit against different peptides (IGP 1273 and 1274) containing the epitope region 158-169. Briefly peptides were adsorbed to microtiterplates as streptavidin complexes. Binding of antibodies was vizualized using a rabbit-anti-human or goat-anti-rabbit antibody conjugated to peroxidase.

DETAILED DESCRIPTION OF THE INVENTION

The invention described herein draws on previously published work and pending patent applications. By way of example, such work consists of scientific papers, patents or pending patent applications. All these publications and applications, cited previously or below are hereby incorporated by reference.

The present invention is based on the finding of a polypeptide derived from HBsAg which competes with the HBsAg/annexin V interaction, or which binds a compound or antibody competing with this interaction, and is immunogenic. Accordingly, this polypeptide, and antibodies against it, can be used to prevent, diagnose or treat HBV and/or HDV infection. In this regard, it should be clear that the usage of the terms "HBV and/or HDV" indicate that an infection with HBV can occur solely or can be accompanied by a superinfection with HDV. On the other hand, an infection with HDV does not occur solely. In other words, the polypeptides of the present invention, and antibodies against them, can be used to prevent, diagnose or treat a HBV infection solely or a mixed HBV/HDV infection.

HBsAg is a known antigen (Heerman et al., 1984; Robinson et al., 1987). Based on this knowledge, polypeptides derived from HBsAg, as herein described, can be prepared by any method known in the art such as classical chemical synthesis, as described by Houbenweyl (1974) and Atherton & Shepard (1989), or by means of recombinant DNA techniques as described by Maniatis et al. (1982). As used herein, the terms "polypeptides derived from HBsAg" refer to polypeptides having an aa sequence which is equal or similar to a part of the aa sequence of

"small" HBsAg. It should be clear that the "polypeptides derived from HBsAg" can be derived from any genotype of HBV (genotype A to F, see Example 6). Furthermore, the term "polypeptide" refers to a polymer of aa which comprises less aa in its sequence than HBsAg, more specifically to a polypeptide which comprises preferentially less than 226, 200, 190, 180, 170, 160, 150, 140, 130, 120, 110, 100, 90 or 80 aa, and most preferably less than 70 aa. This term does not refer to, nor does it exclude, post-translational modifications of the polypeptide such as glycosylation, acetylation, phosphorylation, modifications with fatty acids and the like. Included within the definition are, for example, polypeptides containing one or more analogues of an aa (including unnatural aa's), polypeptides with substituted linkages, mutated versions or natural sequence variations of the polypeptides (corresponding to the genotypes A to F of HBV, as indicated above), polypeptides containing disulfide bounds between cysteine residues, as well as other modifications known in the art.

The polypeptides as set out above are particularly characterized in that they bind anti-idiotypic antibodies (Ab2) which compete with the binding of HBsAg to Annexin V as set out in the Example section or that they bind directly with annexin V.

The expression "which competes with the HBsAg/annexin V interaction or which binds a compound or antibody competing with the HBsAg/annexin V interaction " refers to the description as given by Hertogs et al. (1993 & 1994). It should be clear that this expression also implies that the polypeptide as defined above may interact with, or may bind (both terms can be used interchangeably), annexin V. Furthermore, it should be understood that the expression "a compound or antibody competing with the HBsAg/annexin V interaction" refers to any molecule which is able to compete with the HBsAg/annexin V interaction. More specifically, the latter terms refer to the anti-idiotypic antibodies as described in the Examples section.

The terms "immunogenic polypeptide" relate to the ability of the polypeptide to provoke an immune response such as antibody production. These terms further imply that the polypeptide contains a B cell and /or T cell epitope.

More particularly, the present invention relates to a polypeptide as defined above which comprises preferentially less than 61, 55, 51, 45, 41, 35, 31 or 25 aa, and most preferably less than 21 aa and includes one of the following sequences: KTCTTPAQGN (SEQ ID NO 2, also referred to as "aa 122-131"), or 9, 8, 7, 6, 5 or 4 aa thereof, or, FAKYLWEWASVR (SEQ ID NO 35, also referred to as "aa 158-169"), or 11, 10, 9, 8, 7, 6, 5 or 4 aa thereof, or, TTPAQGN (SEQ

ID 37, also referred to as "aa 125-131) or 6, 5 or 4 aa thereof. More specifically, the present invention relates, with regard to the polypeptides comprising 4 to 9 aa derived from the sequence aa 122-131 (SEQ ID NO 2) as defined above, to peptides with the following aa sequence: aa 122-125, aa 123-126, aa 124-127, aa 125-128, aa 126-129, aa 127-130, aa 128-131, aa 122-126, aa 123-127, aa 124-128, aa 125-129, aa 126-130, aa 127-131, aa 122-127, aa 123-128, aa 124-129, aa 125-130, aa 126-131, aa 122-128, aa 123-129, aa 124-130, aa 125-131, aa 122-129, aa 123-130, aa 124-131, aa 122-130, aa 123-131 and aa 122-131. In this regard, it should be clear that the present invention preferably relates to the peptide aa 125-131 (SEQ ID 37), aa 125-128, aa 125-129 and aa 125-130. Similarly, the present invention relates, with regard to the polypeptides comprising 4 to 11 aa derived from the sequence FAKYLWEWASVR (also referred to as "aa 158-169 (SEQ ID NO 35) as defined above, to peptides with the following aa sequence: aa 158-161, aa 159-162, aa 160-163, aa 161-164, aa 162-165, aa 163-166, aa 164-167, aa 165-168, aa 166-169, aa 158-162, aa 159-163, aa 160-164, aa 161-165, aa 162-166, aa 163-167, aa 164-168, aa 165-169, aa 158-163, aa 159-164, aa 160-165, aa 161-166, aa 162-167, aa 163-168, aa 164-169, aa 158-164, aa 159-165, aa 160-166, aa 161-167, aa 162-168, aa 163-169, aa 158-165, aa 159-166, aa 160-167, aa 161-168, aa 162-169, aa 158-166, aa 159-167, aa 160-168, aa 161-169, aa 158-167, aa 159-168, aa 160-169, aa 158-168 and aa 159-169. Furthermore, it should also be noted that: 1) the polypeptides comprising 4 to 9 aa which are derived from the sequence KTCTTPAQGN (SEQ ID NO 2) and the polypeptides comprising 4 to 11 aa which are derived from the sequence FAKYLWEWASVR (SEQ ID NO 35), and form a binding region to annexin V, can be mapped by any method known in the art such as those described above, and, 2) the binding region to annexin V formed by the latter polypeptides might be a conformational binding region and should thus not be composed of a contiguous aa sequence.

In this regard, the present invention relates to polypeptides as which comprise the sequences: KTCTTPAQGN (SEQ ID NO 2) or TTPAQGN (SEQ ID NO 37), and the sequence FAKYLWEWASVR (SEQ ID 35), or functionally equivalent parts or variants of said sequences. The terms "functionally equivalent parts or variants of said sequences" refer to any variant or fragment of the peptides represented by SEQ ID 2, 35 and 37 which competes with the hepatitis surface antigen/annexin V interaction or which binds a compound or antibody competing with the hepatitis B surface antigen/annexin V interaction. The latter terms do not specifically refer to, not do they exclude, post-translational modifications of the peptides such as glycosylation,

acetylation, phosphorylation, modifications with fatty acids and the like. Included with the definition are, for example, peptides containing one or more analogues of an aa (including unnatural aa's), peptides with substituted linkages, mutated versions or natural sequence variations of the peptides (corresponding to the genotypes of HBV), peptides containing disulfide bounds between cysteine residues, biotinylated peptides as well as other modifications known in the art.

The present invention further relates to a polypeptide, as defined above, which, upon inoculation in a mammalian host, results in the production of antibodies which specifically bind to said polypeptide, in particular to one of the following sequences KTCTTPAQGN (SEQ ID NO 2) or parts thereof, or, TTPAQGN (SEQ ID 37) or parts thereof, or, FAKYLWEWASVR (SEQ ID NO 35) or parts thereof.

It should be noted that we were surprised to find a polypeptide derived from HBsAg which is involved in annexin V binding and is immunogenic because firstly, there was no reasonable expectation of success to discover the annexin V-binding region on HBsAg, and secondly, the peptide as defined above is not immunogenic when presented to the immune system as part of HBsAg but is only immunogenic when presented as a peptide (see Example 3).

The present invention also relates to a combination of a polypeptide as defined above and a negatively charged phospholipid such as phosphatidylserine. The interaction between phosphatidylserine and A-V is demonstrated in the Examples section. The combination of said polypeptide and said negatively charged phospholipid component may be in any possible way known in the art such as for instance in the form of covalently or non-covalently coupled molecules or in the form of liposomes, etc.

The present invention further relates to a polypeptide composition comprising any combination of the above-described polypeptides. The terms "polypeptide composition" refers to any possible mixture of above-described polypeptides with the same or with a different sequence or any possible linkage (covalently or otherwise) between above-described polypeptides with the same or with a different sequence. Examples of the latter polypeptide compositions are simple mixtures, homo -or hetero branched peptides, combinations of biotinylated peptides presented on streptavidin, avidin or neutravidin, chemically cross-linked peptides with or without spacer, condensing peptides and recombinantly produced peptides.

The present invention also relates to a vaccine composition comprising as an active

substance a polypeptide as defined above which can be used as an inoculum to vaccinate humans against infection with HBV and/or HDV or any mutated strain thereof or to therapeutically vaccinate human carriers of HBV and/or HDV or any mutated strain thereof.

The term "a vaccine composition" relates to an immunogenic composition capable of eliciting protection against HBV and/or HDV, whether partial or complete. The term "as an active substance" relates to the component of the vaccine composition which elicits protection against HBVand/or HDV. An active substance (i.e. the polypeptides of the present invention) can be used as such, in a biotinylated form (as explained in WO 93/18054) and/or complexed to *Neutralite Avidin* according to the manufacturer's instruction sheet (Molecular Probes Inc., Eugene, OR). It should also be noted that "a vaccine composition" comprises, in addition to an active substance, a suitable excipient, diluent, carrier and/or adjuvant which, by themselves, do not induce the production of antibodies harmful to the individual receiving the composition nor do they elicit protection. Suitable carriers are typically large slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric aa's, aa copolymers and inactive virus particles. Such carriers are well known to those skilled in the art. Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: aluminium hydroxide, aluminium in combination with 3-0-deacylated monophosphoryl lipid A as described in WO 93/19780, aluminium phosphate as described in WO 93/24148, N-acetyl-muramyl-L-threonyl-D-isoglutamine as described in U.S. Patent N° 4,606,918, N-acetyl-normuramyl-L-alanyl-D-isoglutamine, N-acetylmuramyl-L-alanyl-D-isoglutamyl-L-alanine2(1'2'dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy) ethylamine and RIBI (ImmunoChem Research Inc., Hamilton, MT) which contains monophosphoryl lipid A, detoxified endotoxin, trehalose-6,6-dimycolate, and cell wall skeleton (MPL + TDM + CWS) in a 2% squalene/Tween 80 emulsion. Any of the three components MPL, TDM or CWS may also be used alone or combined 2 by 2. Additionally, adjuvants such as Stimulon (Cambridge Bioscience, Worcester, MA) or SAF-1 (Syntex) may be used. Furthermore, Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA) may be used for non-human applications and research purposes. "A vaccine composition" will further contain excipients and diluents, which are inherently non-toxic and non-therapeutic, such as water, saline, glycerol, ethanol, wetting or emulsifying agents, pH buffering substances, preservatives, and the like. Typically, a vaccine composition is prepared as an injectable, either as a liquid solution or suspension. Solid forms, suitable for solution on, or suspension in, liquid

vehicles prior to injection may also be prepared. The preparation may also be emulsified or encapsulated in liposomes for enhancing adjuvant effect. The polypeptides may also be incorporated into Immune Stimulating Complexes together with saponins, for example Quil A (ISCOMS). Vaccine compositions comprise an immunologically effective amount of the polypeptides of the present invention, as well as any other of the above-mentioned components. "Immunologically effective amount" means that the administration of that amount to an individual, either in a single dosis or as part of a series, is effective for prevention or treatment. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of the individual to be treated (e.g. nonhuman primate, primate, etc.), the capacity of the individual's immune system to mount an effective immune response, the degree of protection desired, the formulation of the vaccine, the treating's doctor assessment, the strain of the infecting HBV, the co-infection status with HDV, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. Usually, the amount will vary from 0.01 to 1000 µg/dose, more particularly from 0.1 to 100 µg/dose. The vaccine compositions are conventionally administered parenterally, typically by injection, for example, subcutaneously or intramuscularly. Additional formulations suitable for other methods of administration include oral formulations and suppositories. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents. It should be noted that a vaccine may also be useful for treatment of an individual, in which case it is called a "therapeutic vaccine".

The present invention further relates to antibodies, or fragments thereof, which specifically bind to a polypeptide as defined above and inhibit binding of said polypeptide to annexin V. As used herein, the term "antibody" refers to polyclonal or monoclonal antibodies. The term "monoclonal antibody" refers to an antibody composition having a homogeneous antibody population. The term is not limiting regarding the species or source of the antibody, nor is it intended to be limited by the manner in which it is made. In addition, the term "antibody" also refers to humanized antibodies in which at least a portion of the framework regions of an immunoglobulin are derived from human immunoglobulin sequences and single chain antibodies as described in U.S. patent N° 4,946,778. As used herein, the term "fragments (of antibodies)" refers to F_{ab}, $F_{(ab)2}$, F_v, and other fragments which retain the antigen binding function and specificity of the parent antibody. The inhibition of binding of said polypeptides to annexin V by

these antibodies can be demonstrated in experiments which are similar to the ones described by Hertogs et al. (1993 and 1994).

The present invention also relates to the usage of said antibodies as a medicament to treat humans infected with HBV and/or HDV or any mutated strain thereof. The term "medicament" refers to a composition comprising an antibody according to the present invention possibly in the presence of suitable excipients known to the skilled man such as saline, Ringer's solution, dextrose solution, Hank's solution, fixed oils, ethyl oleate, 5% dextrose in saline, substances that enhance isotonicity and chemical stability, buffers and preservatives. The "medicament" may be administered by any suitable method within the knowledge of the skilled man. The preferred route of administration is parenterally. In parental administration, the medicament of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with the pharmaceutically acceptable excipients as defined above. However, the dosage and mode of administration will depend on the individual. Generally, the medicament is administered so that the antibody is given at a dose between 1 μg/kg and 10 mg/kg, more preferably between 10 μg/kg and 5 mg/kg, most preferably between 0.1 and 2 mg/kg. Preferably, it is given as a bolus dose. Continuous infusion may also be used. If so, the medicament may be infused at a dose between 5 and 20 μg/kg/minute, more preferably between 7 and 15 μg/kg/minute.

The present invention further relates to the usage of the polypeptide as defined above for use in a method to detect antibodies which are capable of competing with the HBsAg/annexin V interaction and which are present in a biological sample, comprising:

1) contacting the biological sample to be analysed for the presence of HBsAg antibodies with a polypeptide as defined above,

2) detecting the immunological complex formed between said antibodies and said polypeptide.

As used herein, the term "a method to detect" refers to any immunoassay known in the art such as assays which utilize biotin and avidin or streptavidin, ELISA's and immunoprecipitation and agglutination assays. A detailed description of these assays is given in WO 96/13590. In this regard, the present invention also relates to a diagnostic kit for the in vitro determination of antibodies to HBsAg containing: at least one microplate, polypeptides as defined above, appropriate buffer, blocking, and washing solutions which favor binding of the said polypeptides with the antibodies in human serum samples, and appropriate markers which allow to determine

the complexes formed between the antibodies in human serum samples and said polypeptides.

The present invention also relates to the usage of a polypeptide as defined above as a medicament to treat humans infected with HBV and/or HDV or any mutated strain thereof. The term "medicament" refers to a composition comprising a polypeptide according to the present invention possibly in the presence of suitable excipients known to the skilled man. The suitable excipients, route of administration and dosage of the medicament are the same as described above for a medicament comprising an antibody.

The present invention finally relates to the usage of a polypeptide as defined above for use in a method to screen for drugs which block the binding between annexin V and said polypeptide. As used herein, the term "a method to screen for drugs" refers to any assay known in the art suitable for drug screening. In particular, the term refers to any immunoassay as described in WO 96/13590. The term "drug" may refer to any compound targetting or binding a polypeptide as defined above.

The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and can not be construed as to restrict the invention in any way.

EXAMPLES

Example 1: Transfection of the rat FTO2B cells with human annexin V

To demonstrate the involvement of human A-V (hA-V) in the initial step of HBV infection two in vitro cell models, namely primary human hepatocytes and rat hepatoma cells, were used. Human A-V expression in these cells was assessed by Western-blotting and immunocytochemistry using a monoclonal antibody that specifically detects human A-V (monoclonal antibody U4C8). HBV infectability of these cells was determined by two replicative markers, namely by precore/core mRNA detection and by covalently closed circular (ccc) DNA detection.

Firstly, the specificity of the HBV precore/core mRNA detection as a replicative marker was investigated. Precore/core mRNA was detected in HBV infected liver tissue, but not in control liver tissue negative for HBV infection. It was also detected in HBV producing cells derived from the HBV-DNA transfected HepG2.2.15 cell line (Sells et al., 1987), while it was not detected in

PLC/PRF/5 cells. These cells contain an integrated HBV genome and produce HBsAg, but no HBV infectious particles. The HBV inoculum used in further experiments contained no precore/core mRNA, but only HBV-DNA.

The fact that RNA was indeed the template for amplification was confirmed by omission of the RT step or RNAse digestion before cDNA synthesis which resulted in failure of amplification. Southern Blot hybridization using a HBV-DNA specific oligonucleotide probe showed that the PCR products of HBV infected liver tissue and HBV-DNA transfected HepG2.2.15 cells (Sells et al., 1987) contain specific HBV sequences. No band was observed in control liver tissue.

In order to confirm further that precore/core mRNA is the result of HBV replication and not due to a contamination of RNA present in HBV particles, another replicative marker, namely ccc DNA was detected by PCR. Ccc DNA was demonstrated in HBV infected liver tissue and in HBV-DNA transfected HepG2 cells, but not in inoculum.

The presence of ccc DNA is associated with the presence of precore/core mRNA.

To demonstrate the involvement of hA-V in the initial step of HBV infection, we have first investigated the infectability of primary human hepatocytes versus rat hepatocytes.

Immunostaining and western blotting showed that human hepatocytes express hA-V, while rat hepatocytes express no hA-V. After in vitro infection of primary human hepatocytes precore/core mRNA was detected from 1 day to 15 days after infection so far investigated and HBV-DNA became detectable in the culture medium from 3 to 15 days after infection so far investigated. After in vitro infection of rat hepatocytes no precore/core mRNA, neither HBV-DNA was detected.

In order to further determine the involvement of human annexin V in HBV infection, a rat hepatoma FTO2B cell line which is not infectable by HBV and does not express hA-V, was transduced with a construct containing the hA-V gene. One of the transduced cell lines is assigned as FTO9.1. Immunostaining and western blotting showed that the hA-V transfected rat hepatoma cell line FTO9.1 express hA-V, while the original non-transfected rat hepatoma cell line FTO2B does not express hA-V. The infectability of the FTO9.1 cell line was demonstrated by HBV precore/core mRNA detected as early as one day after infection, while non-transduced FTO2B cells were negative for HBV precore/core mRNA up to 7 days after infection so far investigated. In addition, ccc DNA was detected in FTO9.1 cells from 1 day up to 3 day after infection so far investigated. Furthermore, HBV-DNA, which was not detectable in the culture medium before

infection and in the first 2 days after infection, became detectable from day 3 until day 7 so far investigated. HBV-DNA was not detectable in culture medium of non-transduced FTO2B cells up to one week after infection.

We can conclude that human annexin V is involved in the initial step of HBV infection and that human annexin V plays a key role in the cell susceptibility to and the species barrier of HBV infection.

Example 2: The effect of anti-idiotypic antibodies (Ab2) on HBV infection in primary cultures of human hepatocytes.

Human hepatocytes were isolated from human liver tissue obtained from post mortem donors by two-step collagenase perfusion method as described previously (Rijntjes et al., 1986). Cultured human hepatocytes were infected overnight at 37 °C by incubation with a 1/20 dilution of infectious human serum (HBV-DNA positive, HBsAg positive, HBeAg positive) followed by thorough washing. A sandwich ELISA using specific monoclonal antibodies was used both for capturing and detection of HBsAg.

As shown in figure 1, HBsAg production was observed in the culture medium of human hepatocytes infected with HBV containing inoculum (n=5). In contrast, in the presence of Ab2, no such HBsAg production could be observed. Figure 1 further indicates that the production of HBsAg was maximal at day 8 post infection. Thereafter, the HBsAg production declined due to deteriorated conditions of the primary culture of human hepatocytes as has also been seen in parallel control experiments.

Since the presence of HBV replicative intermediates in the infected cells is an indispensable marker for succesful infection, total DNA is isolated from primary cultures of human hepatocytes used for these experiments and subjected for Southern blot analysis (Maniatis et al., 1982). As a control for the specificity of the probe, DNA isolated from the HepG2.2.15 cell line, as defined in *Brief description of Figure 2*, was also investigated. As shown in figure 2, HBV-DNA and replicative intermediates were detected in cells that were infected with inoculum in the absence of Ab2. In contrast, HBV-DNA sequences were not observed in experiments in which Ab2 were used.

Example 3: Mapping of the annexin V-binding site on HBsAg

To find the binding site on HBsAg the region composed of aa 112 to 169 was investigated. Firstly, because this region has been predicted to be located on the outer surface of the virus; Secondly, because binding of HBsAg with anti-idiotypic antibody Ab2 (generated in rabbits immunized with human Annexin V, Hertogs et al., 1994) is completely inhibited after digestion of HBsAg with endoproteinase-LysC. Lysines are only found in this region of the HBsAg molecule (genotype A), namely at aa 122, at aa 141 and at aa 160. Three peptides covering this region were synthesized. The first two peptides represent the outer part of the HBsAg molecule. These peptides have been derived from a genotype C sequence. The third polypeptide represents the region of the outer part of HBsAg which is most subjected to non-conservative mutations. This peptide is derived from genotype A.

polypeptide IGP673 (aa 112-141): GTGTTSTGPCKTCTIPAQGTSMFPSCCCTK (genotype C)
polypeptide IGP671 (aa 139-169): CTKPSDGNCTCIPIPSSWAFARFLWEGASVR (genotype C)
polypeptide IGP80 (aa 122-157): KTCTTPAQGNSMFPSCCCTKPTDSNCTCIPIPSSWA (genotype A)

Since Ab2 binds polypeptide 3, we concluded that this polypeptide contains the binding epitope on HBsAg for human annexin V.

Based on these results and since monoclonal antibodies recognizing the HBsAg major "a" determinant covering amino acid positions 139 to 147 do not interfere with the HBsAg/annexin V interaction, the "a" determinant may not be directly involved in annexin V binding. Therefore, we focused on the amino terminal part of the HBsAg ectodomain. Polypeptides covering the region between aa 99 and 136 of "small" HBsAg (IGP 1076-1083, see table 1) were synthesized. The polypeptides 1077 and 1080, which are derived from genotype C, comprised a mutation (aa 107 from C to S) in order to reduce the number of cysteines in the peptide. Moreover, there are two aa within the region aa 99-136 which appear to mutate in a non-conservative way (eg. aa 126 from T to I and aa 131 from N to T) and which determine four of the naturally occuring genotypes of HBsAg (A, B, C and D, see table 1). For this reason, we synthesized two other peptides: one bearing the sequence aa 115-134 from genotype A (IGP 1094) and another one containing the genotype C mutation at position 126 only C (IGP 1093). In this respect, it should

be noted that some other natural mutations also exist but are usually of a conservative nature (aa 122 from R to K, aa 134 from F to Y and aa 113 and 114 from S to T).

All these synthesized polypeptides (IGP 1076-1094) were tested for reactivity with the anti-idiotypic antibody Ab2 (see above *Brief description of drawings and tables*). Ab2 mimics annexin V and bound IGP 1094 only (figure 3). Polypeptide 1094 was also produced a second time and the latter result was confirmed a second time.

The lack of reactivity of the other polypeptides covering the region 115-134 was found to be a consequence of substitutions at positions 126 and 131. Indeed, four polypeptides based on IGP 1094 were synthesized representing all the combinations of mutations at these two positions (IGP 1102-1105). The latter polypeptides were synthesized in a biotinylated form and purified by RP-HPLC. Subsequently, their reactivity with Ab2 was determined (see figure 4). As such, we demonstrated that binding of peptides to Ab2 is indeed influenced by the latter mutations: the mutation at position 126 (T to I, peptide IGP 1103 and 1102) resulted in a minor loss of binding whereas the mutation at position 131 (N to T, peptides IGP 1104 and 1105) resulted in an almost complete loss of binding to Ab2.

It therefore appears that the C terminal region of this polypeptide (aa 125-134) is highly important since mutations within this region can alter or destroy binding with Ab2. Within the complete HBsAg molecule, however, these mutations do not destroy binding to annexin V since binding of annexin V and Ab2 to HBsAg of either genotypes A or D occurs (see figure 5) possibly because the complete molecule adopts a more rigid conformation which is not affected by mutations at positions 126 and 131. On the other hand, within a short polypeptide (20 aa) a single mutation may result in more drastic conformational changes which influences binding to annexin.

Example 3': Mapping of the annexin V-binding site on HBsAg (further experiments)

To further characterize which part of the polypeptide 115-134 is responsible for binding of HBsAg, peptides of 10 amino acids covering the sequence were subsequently synthesized.

pept (115-134) TTSTGPCKTCTTPAQGNSMF (SEQ ID 1)

P461 (115-124) TTSTGPCKTC (SEQ ID 25)

P462 (116-125)	TSTGPCKTCT	(SEQ ID 26)
P463 (117-126)	STGPCKTCTT	(SEQ ID 27)
P464 (118-127)	TGPCKTCTTP	(SEQ ID 28)
P465 (119-128)	GPCKTCTTPA	(SEQ ID 29)
P466 (120-129)	PCKTCTTPAQ	(SEQ ID 30)
P467 (121-130)	CKTCTTPAQG	(SEQ ID 31)
P468 (122-131)	KTCTTPAQGN	(SEQ ID 2)
P469 (123-132)	TCTTPAQGNS	(SEQ ID 32)
P470 (124-133)	CTTPAQGNSM	(SEQ ID 33)
P471 (125-134)	TTPAQGNSMF	(SEQ ID 34)

These peptides were tested for reactivity with anti-idiotypic antibody Ab2. We found that peptide P468, which covers the region of aa 122 to aa 131 of HBsAg, is able to bind anti-idiotypic antibody (Ab2). This means that the binding epitope of HBsAg must be located in the region from aa 122 to aa 131. Since Ab2 have previously been shown to compete the binding of HBsAg to A-V, these experiments indicate that the Annexin V binding domain of HBsAg is located between aa 122 and aa 131, or in the conformational vicinity of this region.

Therefore, the skilled man may synthesize variants and/or derivatives of said region which specifically bind human Annexin V. Such variants may include any types of peptide variations discussed above.

Example 4: Influence of phosphatidylserine on the HBsAg-annexin V binding

The propensity of annexin V to bind phospholipids (phosphatidylserine) has led also to consider the possibility that this protein might bind to HBsAg via its lipid content, since HBsAg lipids may account for up to 30% of the mass of HBsAg particles. Herefore, the influence of phospholipids on annexin V binding to HBsAg was investigated. A clear inhibiting effect of phosphatidylserine on the binding of A-V to HBsAg is observed, while phosphatidylcholine did not inhibit the binding (see figure 6). Moreover, HBV infection of transfected rat hepatoma cells, namely FTO9.1 cells can be inhibited by phosphatidylserine.

To further elucidate the binding mechanism between HBsAg and annexin V, we used monoclonal

antibodies directed against human annexin V. All mAbs are of the IgG class. Two mAbs, namely U4C8 and CM1995 (which can be obtained from Dr. Erik Depla, Innogenetics N.V., Industriepark 7 box 4, B-9052 Ghent, Belgium; phone: +32 9 241 07 11; fax: +32 9 241 07 99), were found to inhibit the binding of annexin V to HBsAg (see figure 7). The binding of labelled annexin V to a monoclonal antibody is reduced by unlabelled human liver annexin V, but not by rat liver annexin V (see figure 8). This means that these antibodies are species specific. Mutual competition between these two antibodies revealed that they do not compete with each other for binding to annexin V, which means that they recognize different epitopes. The third antibody U1E10 (which can be obtained from Dr. Erik Depla, Innogenetics N.V., Industriepark 7 box 4, B-9052 Ghent, Belgium; phone: +32 9 241 07 11; fax: +32 9 241 07 99), is not species specific and is not able to compete with HBsAg for binding to annexin V, moreover this antibody does not compete with U4C8 and CM1995 and recognizes therefore a third epitope on A-V.

The influence of phospholipids on the binding of these mAbs to annexin V was subsequently investigated (see figure 9). From the finding that phosphatidylserine can inhibit the binding of CM1995 to annexin V, but only very little the binding of U4C8to annexin V, we can conclude that CM1995 inhibits a protein-phospholipid interaction, while U4C8 inhibits a protein-protein interaction between human annexin V and HBsAg. Moreover the interaction of HBsAg with Ab2 is not influenced at all by phospholipids of any kind.

We can conclude that HBsAg-annexin V binding is based on at least a dual interaction. This includes binding of annexin V to negatively charged phospholipids, such as phosphatidylserine, of the HBsAg particle via the phospholipid binding site of annexin V, and a second interaction which is based upon a protein to protein binding between a second distinct epitope of annexin V and the protein content of the HBsAg particle. Figure 10 gives an overview of this interaction.

Example 5: Immunogenicity of the sequence aa 115-134.

The annexin V binding domain of HBsAg was produced both as an N-terminally biotinylated polypeptide (IGP 1103), purified by RP-HPLC (96.6% purity), and as a tetrameric polypeptide on a lysine matrix which was biotinylated C-terminally (IGP 1119, see table 1). The sequence of peptide 1119 was further elongated with another 2 aa at the C-terminus in order to obtain a small linker between the lysine matrix and the sequence of interest. Also the latter peptide

was purified by RP-HPLC in order to remove chemicals resulting from peptide synthesis. Of both peptides a *Neutralite Avidin* complex (Molecular Probes, Inc., Eugene, OR) was made.

-peptide 1103: 200 µg of peptide was mixed with 1.1 mg of *Neutralite Avidin* in a final volume of 2.5 ml saline. This solution was stored at -20°C as 500 µl aliquots for vaccine purposes.

-peptide 1119: 832 µg of peptide was mixed with 1.1 mg of *Neutralite Avidin* in a final volume of 2.5 ml saline. This solution was also stored at -20°C as 500 µl aliquots for vaccine purposes.

Both the free peptides and the two *Neutralite Avidin* complexes, as described above, were evaluated for binding with Ab2. All results were positive (i.e. all peptides did bind to Ab2) and a chimp was immunized according to the following protocol: *Neutralite Avidin*-complexed peptides were mixed with an equal volume of reconstituted RIBI (ImmunoChem. Research Inc., Hamilton, MT) as an adjuvant before injection into one of the upper arms of the chimpanzee; the injections were performed on days 0 and 21; bleedings were taken on day -3, 4, 11, 18, 25 and 32 post-immunization.

Antibody response towards both *Neutralite Avidin*-complexed peptides, two control peptides (i.e. IGP 1038 and 1030 with similar characteristics [solubility, biotinylation, branching] as IGP 1103 and 1119, respectively) and HBsAg were monitored. A clear and specific reactivity was observed with HBsAg and with the peptides IGP 1103 and 1119 (Figure 11). In addition, the Ab titers against both HBsAg and the peptides IGP 1103 and 1119 increased after the second immunization (Figure 12). These results, thus, demonstrate that the peptide sequence aa 115-134 bears a B-cell epitope which is also present on HBsAg since Ab's induced by immunization with aa 115-134 specifically recognize HBsAg.

A control chimpanzee was also challenged with a standard inoculum for HBV. Serum samples were taken before and after inoculation and were screened for the presence of antibodies binding to HBsAg and to the peptides IGP 1103 and IGP 1119. The specificity of binding was verified using the same control peptides as indicated above (IGP 1030 and 1038). Although anti-HBsAg seroconversion could be clearly shown, no specific antibody reactivity against any of the peptides (IGP 1103, 1119, 1030 and 1038) could be demonstrated (Figure 13). These results demonstrate that the B-cell epitope within the region aa 115-134 of HBsAg may not be particularly immunogenic when presented to the immune system as part of HBsAg.

Example 6: Cross-reactivity of antibodies evoked by a genotype A derived peptide.

The serum of a chimpanzee immunized with peptides 1103 and 1119 was evaluated for the presence of antibodies cross-reacting with the same region of HBsAg (aa 115-134) but derived from other genotypes. As can be judged from figure 14, the antibody response evoked by the genotype A derived peptides 1119 and 1103 clearly cross-reacts with peptides carrying the non-conservative mutations from genotype B (IGP 1104), genotype C (IGP 1105) and genotype D (IGP 1104). As a consequence it may be concluded that the peptides derived from the same region of HBsAg (aa 115-134) but from another genotype also will evoke a cross-reactive immune response, similar to the one evoked by the genotype A derived peptides IGP 1103 and 1119.

Example 7: Direct binding of annexin V to HBsAg peptides representing the amino acid region 112-169.

Binding of annexin V (coupled with horse radish peroxidase) to HBsAg itself or to biotinylated peptides coated to microtiterplates as a streptavidin complex was evaluated. These peptides represent the region 112-169 of HBsAg (IGP 671, 673 and 80), the peptide binding with the anti-idiotypic antibody (IGP 1119) as well as a control peptide (IGP 1038). Binding specificity is demonstrated by using an excess (100 µg/ml) of an anti-annexin V monoclonal antibody (U4C8, see example 4) which is known to compete with the binding of annexin V to HBsAg. The complete binding assay is performed in a tris buffered saline (10 mM tris, 150 mM NaCl, pH 7.5) to which 1mM $CaCl_2$ and 1mM $MgCl_2$ are added (=$TBS_{Ca/mg}$). After coating of HbsAg or peptide/streptavidin complexes to the plate, aspecific binding is reduced by blocking the plates with $TBS_{Ca/mg}$-3% gelatin at 37°C (fish gelatin). The incubation with annexin V and eventual competitors is performed in $TBS_{Ca/mg}$-0.3% gelatin at 37°C. After incubation plates are washed with $TBS_{Ca/mg}$-0.05% Tween 20.

The data shown in Figure 15 demonstrate that the peptides IGP 671 and IGP 1119 bind to annexin V. The latter data confirm the importance of the amino acid region 115-134 as covered by IGP 1119, but also demonstrate the importance of the region aa 158-169 since IGP 671 which covers the latter aa region binds to annexinV.

Example 8: Further fine mapping of the annexin V binding site on HBsAg

Based on the results from example 7, a new series of peptides was synthesized (IGP 1189-1193). Their sequences cover the potential epitopes for binding to annexin V as concluded from examples 3, 3' and 7. In addition, the potential epitopes were combined in one molecule as hetero-branched peptides (IGP 1190-1192). The setup of the experiment is similar as for example 7 and again the specificity was demonstrated by competing the binding to annexin V with the same monoclonal antibody (see Figure 16).

The highest binding was observed with the peptides IGP 1193, 1190, 671 and 1119. It therefore appears that the sequence of IGP 1119 (aa 115-134), and more specifically the amino terminal part of this sequence which is represented by IGP 1193 (aa 115-125), plays a crucial role in the binding of annexin V to HBsAg. However, also the C-terminal part of IGP 1119, especially in combination with the amino acid region 158-169, appears important in the binding of annexin V to HBsAg. The latter is demonstrated by the binding of the hetero-branched peptide IGP 1190 with annexin V in this example and the binding of Ab2 to the peptide covering amino acid region 122-131 shown in example 3'.

Example 9: Further fine mapping of the annexin V binding site on HBsAg by direct competition.

When the series of peptides used in example 8 were analysed (in an assay similar to the one outlined in example 7) for their ability to compete with the binding of HBsAg to annexin V, only peptides IGP 1119 and 1193 were found to do so in the concentration range studied. Peptides were presented as streptavidin-complexes in order to improve their presentation in solution. In the present experiment, binding of annexin V to HBsAg coated on microtiterplates was competed with HBsAg or with the peptides IGP 1119 and IGP 1193 in solution (both peptides were presented as streptavidin complexes). The results (see Figure 17, molar excess = [competitor in solution]/[HBsAg coated]) confirm the importance of the amino acid region 115-134, and especially the N-terminal part of this region as covered by IGP 1193.

Example 10: Further fine mapping of an Ab2 epitope.

The peptides mentioned under example 3' (P461-P471) were further analyzed for their capacity to bind Ab2 using a different approach as used in example 3'. In the latter example peptides were adsorbed to microtiterplates and the binding with Ab2 was evaluated. This may result in false negative scoring since not all of these peptides will adsorb equally well to the microtiterplate. In the current setup, HBsAg itself is adsorbed to the microtiterplate and the peptides are used in solution. Ab2 is allowed to bind to the peptides or HBsAg and finally binding of Ab2 to HBsAg itself is evaluated. In this way partial competition was observed with the peptides P468, P469, P470 and P471 (Figure 18, only the results for the peptide P467-471 are shown). This result allows to further map the binding site (or at least one of the binding sites) of Ab2 to the region 125-131 (TTPAQGN), which is the common part of these peptides. This result confirms the importance of the amino acids 126 and 131 in this Ab2 epitope. These two amino acids determine the genotype specific reactivity of this Ab2. The binding of Ab2 is however only partially genotype specific, since good reactivity is also observed with genotype D (example 3, figure 5). This observation, together with the finding of a 'partial' (up to 50%, figure 18) competition with the peptides P468-471 leads to the conclusion that another epitope must be involved in Ab2 (and consequently of annexin V) binding to HBsAg. This is also supported by the finding that the A-V binding is not genotype specific (example 3, figure 5). Based on the previous experiments two candidate regions have been identified:

region 1 aa 115-126
region 2 aa 158-169

Example 11: Further establishment of the role of region 115-126.

In the light of the previous results the binding of annexin V to IGP 1193 (aa 115-126) needs confirmation since this peptide overlaps only with 2 amino acids with the Ab2 epitope, and may therefore be the second epitope needed to cover for complete Ab2/annexin V binding. Moreover this region does not carry amino acids which change in a non-conservative way according to the genotype.

In order to confirm the results previously obtained (example 8 and 9) with this peptide purified peptide was needed. The crude peptide was first evaluated on mass-spectrometry (MS). The spectrum revealed a whole series of molecules, however none of them of the expected size and over

90% of the peptides having a molecular weight substantially higher then expected (data not shown). For these reasons this peptide was synthesized again and immediately analyzed by MS. The new peptide preparation, which will be referred to as IGP 1193B, contained the expected molecule, however no binding with annexin V was. The lack of binding of annexin V to the region 115-126 was further confirmed by a whole series of new peptides which represent this region in different ways of presentation (branched peptide: IGP 1363, C-terminal biotinylation: IGP 1368).

IGP 1193B	bio-TTSTGPCKTCT
IGP 1363	$(TTSTGPCKTCT)_2$-KKGK(bio)GA
IGP 1368	TTSTGPCKTCT-KKGK(bio)GA

This result is also confirmed from another experimental approach. A monoclonal antibody, C11F5, which is directed against HBsAg but is not able to inhibit the binding of annexin V to HBsAg was mapped (example 12). Part of the epitope of this antibody covers the region 119-124, which is an additional prove that this region is not involved in Ab2 and thus in annexin V binding.

Example 12 : Mapping of other epitopes (monoclonal antibodies).

Using our complete series of peptides from the HBsAg ectodomain, mapping of other determinants, such as monoclonal antibody epitopes, was pursued. Of the monoclonal antibodies investigated, only one, C11F5 (which can be obtained from Dr. Erik Depla, Innogenetics N.V., Industriepark 7 box 4, B-9052 Ghent, Belgium; phone: +32 9 241 07 11; fax: +32 9 241 07 99), could be mapped in detail. The reactivity of this monoclonal to all the peptides used is shown in figure 19, while the relative (to HBsAg) competition of the purified peptides 1076-1083 for binding of C11F5 to HBsAg is shown in Table 2. The peptides 1076 and 1081 show both a higher relative competition, compared to the other peptides. This observation supports the existence of the first part of the epitope in the region 105-111. Mutation of the cysteine at position 107 results in a loss of relative affinity which is comparable with the loss if this part of the epitope is omitted. The second part of the epitope is found in the region 119-124 (reactivity with peptide 1083, 1193B and 1275). This combined epitope is surprising, since it has never been described before and especially since it does not fit with the current structural model of HBsAg as predicted by Chen *et al.*, 1996.

Therefore the mapping of this epitope leads to new insights in the structure of HBsAg.

Example 13 : Further elaboration of the role of the region 158-169.

The role of this part of HBsAg was further evaluated using a series of new peptides (1273, 1274, 1362, 1367). The binding of annexin V to these peptides is shown in figure 20. For this experiment binding was checked for all peptides containing the 158-169 region and some additional control peptides also derived from HBsAg. This assay was performed at room temperature (and not 37°C as in the previous examples) in order to select for peptides showing a high binding. The interpretation of these results indicate that :

1/ the binding previously observed to the peptides 671 and 1190 is confirmed;

2/ the region 158-167 contains sufficient information to allow binding of annexin V: binding to peptides 1367 and 1362;

3/ the N-terminus of this region needs to be available for binding since only the C-terminal biotinylated peptide allows binding, this observation stresses the importance of correct presentation of an epitope, the N-terminal biotinylated peptide 1274 does not allow binding of annexin V. This finding stresses the importance of the way of presentation of a peptide in order to allow reactivity. This was confirmed in competition experiments (figure 21) which reveal the superior presentation of the 158-169 epitope by the peptide IGP 1362. From these experiments it can be concluded that the presentation will also be crucial in order to obtain good immune reactivity.

Example 14 : Immunogenicity.

The peptides 1119 and 1273 and 1274 were used for immunization of rabbits. As for the chimpanzee immunization (example 5) the peptides were complexed with Neutralite avidin. Of each peptide three times 100 µg peptide complexed with an equimolar amount of Neutralite avidin was injected. In total six rabbits were used (2 rabbits for each peptide).

The antibody responses were evaluated for cross-reactivity with HBsAg and were mapped using all available peptides from the previous examples. For peptide 1119 the induced immune response was compared to the immune response induced in chimpanzee by a mixture of IGP 1103 and 1119 (example 5). The immune response induced in rabbits by 1273 (115-126 and 158-169 as branched

peptide) and 1274 (158-169 as N-terminal biotinylated peptide) were also compared as both peptides contain the sequence 158-169 but in a completely different presentation.

From figure 22 it is clear that the immunization of a chimpanzee and rabbit results in an effective immune response against the sequence of the complete antigen (IGP 673, 1102, 1103, 1104, 1105, 1119 and HBsAg itself) however the recognition of single epitopes is completely different. No antibodies directed against the Ab2 epitope were detected in the chimpanzee (eg lack of reactivity with IGP 1189 and 80). However, both rabbits immunized with IGP 1119 solely mounted a very effective immune response against this specific epitope. The major conclusion from this experiment may be that IGP 1119 is much more efficient in generating the desired immune response, the simultaneous immunization with IGP 1103 resulted in an altered immune response with recognition of other epitopes. From this example it can also be predicted that a peptide limited to the desired epitope (122-131 or 125-131) will even be much more effective in generating the desired response since such peptide allows no alternative reactivity which were clearly selected in the chimpanzee experiment. In addition the presentation of such a minimal epitope will be crucial as can be judged from the immunizations with IGP 1273 and 1274.

When comparing the rabbit immune responses against IGP 1273 and 1274 it is immediately clear (Figure 23) that IGP 1274 fails to mount an efficient immune response, this can be judged from the lack of reactivity against HBsAg and the very poor reactivity with the peptides. However, the immunization with IGP 1273 results in a very effective immune response. Reactivity against HBsAg is observed and all peptides carrying the sequence 158-169 are recognized, however the sequence 115-126 which is also part of this peptide did not generate antibodies (eg lack of reactivity with IGP 1193B, 1363 and 1368). This example shows again that the structure and way of presentation is crucial in order to mount the desired immune response. From these examples it can be stated that any man skilled in the art may engineer such molecules using by way of example, but not limited to, peptides which are branched (homo- or hetero- (heterobranching can be performed by any sequence which is HBsAg derived or not), which are biotinylated (N-terminal with or without spacer, C-terminal with or without spacer) and complexed to avidin like molecules, which are covalently conjugated to non-related molecules (eg ovalbumin, hemocyanin), which are repeats of a single sequence, which are made cyclic (eg addition of cysteine at C- and N-terminus).

Taken together, the above-described experiments clearly demonstrate that the amino acid regions

122-131 and 158-169, or parts thereof, are directly involved in the binding of HBsAg to annexin V. Any new molecule which comprises these regions, or variants or parts thereof, and which specifically binds human annexin V is therefore part of the present invention.

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Table 1

```
                100       110       120       130       140       150       160       170
            geno *         *         *         *         *         *         *         *
HBsAg       A   DYQGMLPVCPLIPGSTTTSTGPCKTCTTPAQGNSMFPSCCCTKPTDGNCTCIPIPSSWAFAKYLWEWASVR (SEQ ID 38)
HBsAg       B   DYQGMLPVCPLIPGSSTTSTGPCKTCTTPAQGTSMFPSCCCTKPTDGNCTCIPIPSSWAFAKYLWEWASVR (SEQ ID 39)
HBsAg       C   DYQGMLPVCPLLPGTSTTSTGPCKTCTIPAQGTSMFPSCCCTKPSDGNCTCIPIPSSWAFARFLWEWASVR (SEQ ID 40)
HBsAg       D   DYQGMLPVCPLIPGSSTTSTGPCRTCTTPAQGTSMYPSCCCTKPSDGNCTCIPIPSSWAFGKFLWEWASAR (SEQ ID 41)
IGP 673                  bio-GTGTTSTGPCKTCTIPAQGTSMFPSCCCTK (SEQ ID 3)
IGP 671                                             bio-CTKPSDGNCTCIPIPSSWAFARFLWEGASVR (SEQ ID 4)
IGP 80                             bio-KTCTTPAQGNSMFPSCCCTKPTDSNCTCIPIPSSWA (SEQ ID 5)
IGP 532                                             bio-CTKPSDRNCTCIPIP (SEQ ID 42)
IGP 533                                             bio-CTKPSDGNCTCIPIP (SEQ ID 43)
IGP 534                            bio-KTCTIPAQGTSMFPSC (SEQ ID 44)
IGP 1076              PVCPLLPGTSTTSTGPCKTCTIPAQGTSMFPS (SEQ ID 6)
IGP 1077              PVSPLLPGTSTTSTGPCKTCTIPAQGTSMFPS (SEQ ID 7)
IGP 1078                     GTSTTSTGPCKTCTIPAQGTSMFPS (SEQ ID 8)
IGP 1079                            GPCKTCTIPAQGTSMFPS (SEQ ID 9)
IGP 1081        DYQGMLPVCPLLPGTSTTSTGPCKTCTIPAQG (SEQ ID 10)
IGP 1080        DYQGMLPVSPLLPGTSTTSTGPCKTCTIPAQG (SEQ ID 11)
IGP 1082                 PLLPGTSTTSTGPCKTCTIPAQG (SEQ ID 12)
IGP 1083                            GPCKTCTIPAQG (SEQ ID 13)
IGP 1093                        TTSTGPCKTCTIPAQGNSMF (SEQ ID 14)
IGP 1094                        TTSTGPCKTCTTPAQGNSMF (SEQ ID 15)
IGP 1102                    bio-TTSTGPCKTCTIPAQGNSMF (SEQ ID 14)
IGP 1103                    bio-TTSTGPCKTCTTPAQGNSMF (SEQ ID 15)
IGP 1104                    bio-TTSTGPCKTCTTPAQGTSMF (SEQ ID 18)
```

Table 1 con't

IGP 1105	bio-TTSTGPCKTCTIPAQGTSMF (SEQ ID 19)
IGP 1106	TTSTGPCKTCTTPAQGTSMF (SEQ ID 18)
IGP 1107	TTSTGPCKTCTIPAQGTSMF (SEQ ID 19)
IGP 1119	((TTSTGPCKTCTTPAQGNSMFPS)$_2$K)$_2$-KGK(bio)GA (SEQ ID 45)
IGP 1189	(KTCTTPAQGNSM)$_2$-KKGK(bio)GA (SEQ ID 46)
IGP 1363	(TTSTGPCKTCT)$_2$-KKGK(bio)GA (SEQ ID 47)
IGP 1362	(FAKYLEWASVR)$_2$-KKGK(bio)GA (SEQ ID 48)
IGP 1190	KTCTTPAQGNSM------(KKGK(bio)GA)-----FAKYLWEWASVR (SEQ ID 49)
IGP 1191	DYQGMLPVCPL-(KKGK(bio)GA)-KTCTTPAQGNSM (SEQ ID 50)
IGP 1192	DYQGMLPVCPL-----------------(KKGK(bio)GA)-----------------FAKYLWEWASVR (SEQ ID 51)
IGP 1273	TTSTGPCKTCT---------(KKGK(bio)GA)---------FAKYLWEWASVR (SEQ ID 52)
IGP 1193	Bio-TTSTGPCKTCT (SEQ ID 53)
IGP 1275	Bio-TTSTGPCKTC (SEQ ID 25)
IGP 1276	Bio-TTSTGPCKT (SEQ ID 54)
IGP 1277	Bio-TTSTGPCK (SEQ ID 55)
IGP 1278	Bio-TTSTGPC (SEQ ID 56)
IGP 1368	TTSTGPCKTCT-KKGK(bio)GA (SEQ ID 57)
IGP 1274	bio-FAKYLEWASVR (SEQ ID 35)
IGP 1279	bio-AKYLEWASVR (SEQ ID 58)
IGP 1280	bio-KYLEWASVR (SEQ ID 59)
IGP 1281	bio-YLEWASVR (SEQ ID 60)
IGP 1367	FAKYLWEWASVR-KKGK(bio)GA (SEQ ID 61)

control peptides

Table 1 con't

IGP 1038	Bio-GGSQLFTISPRRHETVQD (SEQ ID 20)
IGP 1030	((SVRVEQVVKPPQK)$_2$K)$_2$-KGK(bio)GA (SEQ ID 62)

Table 2

peptide	sequence	Relative competition (HBsAg gives a competition of 1)
IGP 1076	PVCPLLPGTSTTSTGPCKTCTIPAQGTSMFPS	12.9
IGP 1077	PVSPLLPGTSTTSTGPCKTCTIPAQGTSMFPS	0.4
IGP 1078	GTSTTSTGPCKTCTIPAQGTSMFPS	0.3
IGP 1079	GPCKTCTIPAQGTSMFPS	0.5
IGP 1081	DYQGMLPVCPLLPGTSTTSTGPCKTCTIPAQG	8.7
IGP 1080	DYQGMLPVSPLLPGTSTTSTGPCKTCTIPAQG	0.9
IGP 1082	PLLPGTSTTSTGPCKTCTIPAQG	0.4
IGP 1083	GPCKTCTIPAQG	1.0

CLAIMS

1. An immunogenic polypeptide derived from hepatitis B surface antigen which competes with the hepatitis B surface antigen/annexin V interaction or which binds a compound or antibody competing with the hepatitis B surface antigen/annexin V interaction.

2. A polypeptide according to claim 1 which comprises less than 61 amino acids containing at least 4 amino acids of one of the following sequences:

-FAKYLWEWASVR (SEQ ID 35)

- KTCTTPAQGN (SEQ ID 2)

- TTPAQGN (SEQ ID 37).

3. A polypeptide according to claim 1 which comprises the sequence KTCTTPAQGN (SEQ ID 2) or TTPAQGN (SEQ ID 37), and, the sequence FAKYLWEWASVR (SEQ ID 35) or functionally equivalent parts or variants of said sequences.

4. A polypeptide according to claims 2 and 3 or any mutated version thereof which, upon inoculation in a mammalian host, results in the production of antibodies which specifically bind to said polypeptide, in particular to the sequence KTCTTPAQGN (SEQ ID 2) or a part thereof, or, to the sequence TTPAQGN (SEQ ID 37) or a part thereof, or, to the sequence FAKYLWEWASVR (SEQ ID 35) or a part thereof.

5. A combination of a polypeptide according to any of the previous claims and a negatively charged phospholipid.

6. A polypeptide composition comprising any combination of polypeptides according to claims 2-5.

7. A vaccine composition comprising as an active substance a polypeptide as defined in any of claims 1 to 4.

8. A vaccine composition according to claim 7 for use as an inoculum to vaccinate humans against infection with hepatitis B virus and/or hepatitis delta virus or any mutated strain thereof or to

therapeutically vaccinate human carriers of hepatitis B virus and/or hepatitis delta virus or any mutated strain thereof.

9. Antibodies which specifically bind to a polypeptide as defined in any of claims 1 to 4 and inhibit binding of HBsAg to annexin V.

10. A pharmaceutical composition comprising as an active substance antibodies, or fragments thereof, according to claim 9 for use in a method to treat humans infected with hepatitis B virus and/or hepatitis delta virus or any mutated strain thereof.

11. A polypeptide according to any of claims 1 to 4 for use in a method to detect antibodies which are capable of competing with the hepatitis B and/or hepatitis delta surface antigen/annexin V interaction and which are present in a biological sample, comprising:
a) contacting the biological sample to be analysed for the presence of HBsAg antibodies with a polypeptide as defined above,
b) detecting the immunological complex formed between said antibodies and said polypeptide.

12. A kit for the in vitro determination of antibodies in human serum containing: at least one microplate, polypeptides according to any of claims 1 to 4, appropriate buffer, blocking, and washing solutions which favor binding of the said polypeptides with the antibodies in human serum samples, and appropriate markers which allow to determine the complexes formed between the antibodies in human serum samples and said polypeptides.

13. A polypeptide according to any of claims 1 to 4 for use as a medicament to treat humans infected with hepatitis B virus and/or hepatitis delta virus or any mutated strain thereof.

14. A polypeptide according to any of claims 1 to 4 for use in a method to screen for drugs which block the binding between annexin V and said polypeptide.

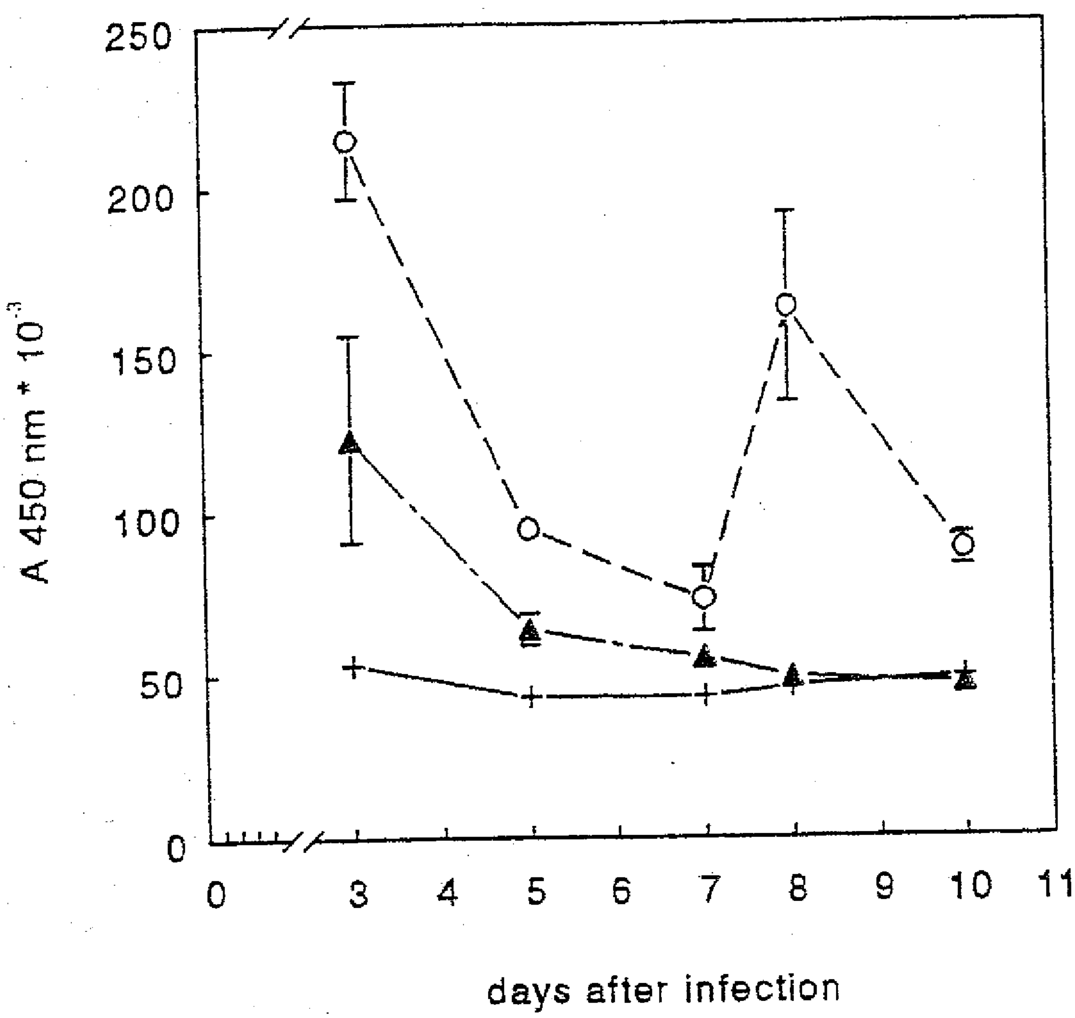

A 450 nm * 10^{-3}
250
200
150
100
50
0
0 3 4 5 6 7 8 9 10 11
days after infection

Figure 1

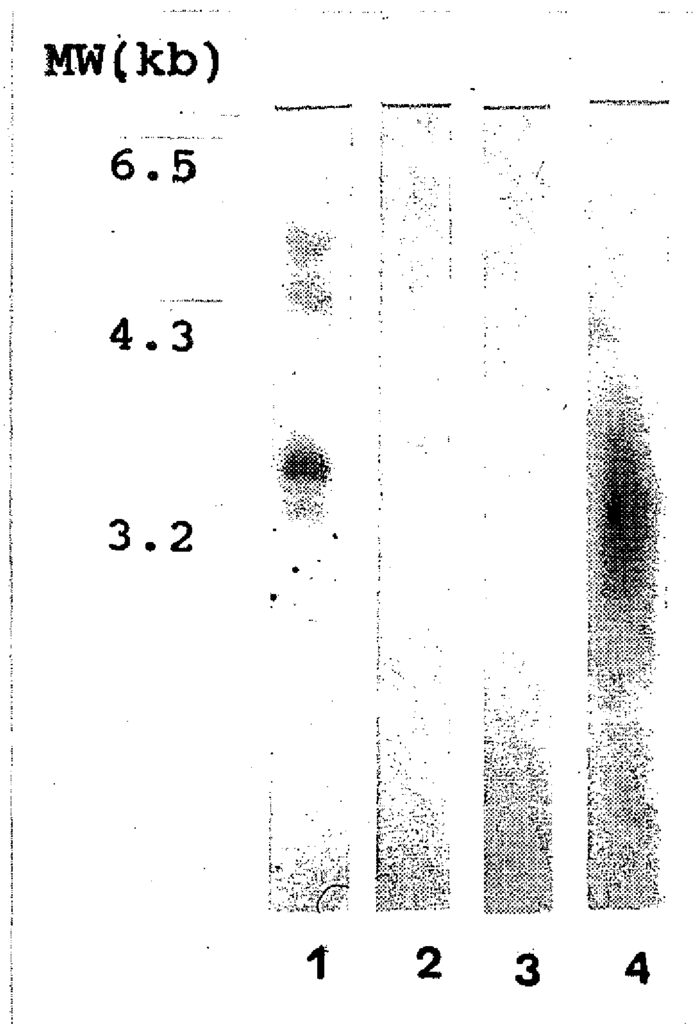


Figure 2

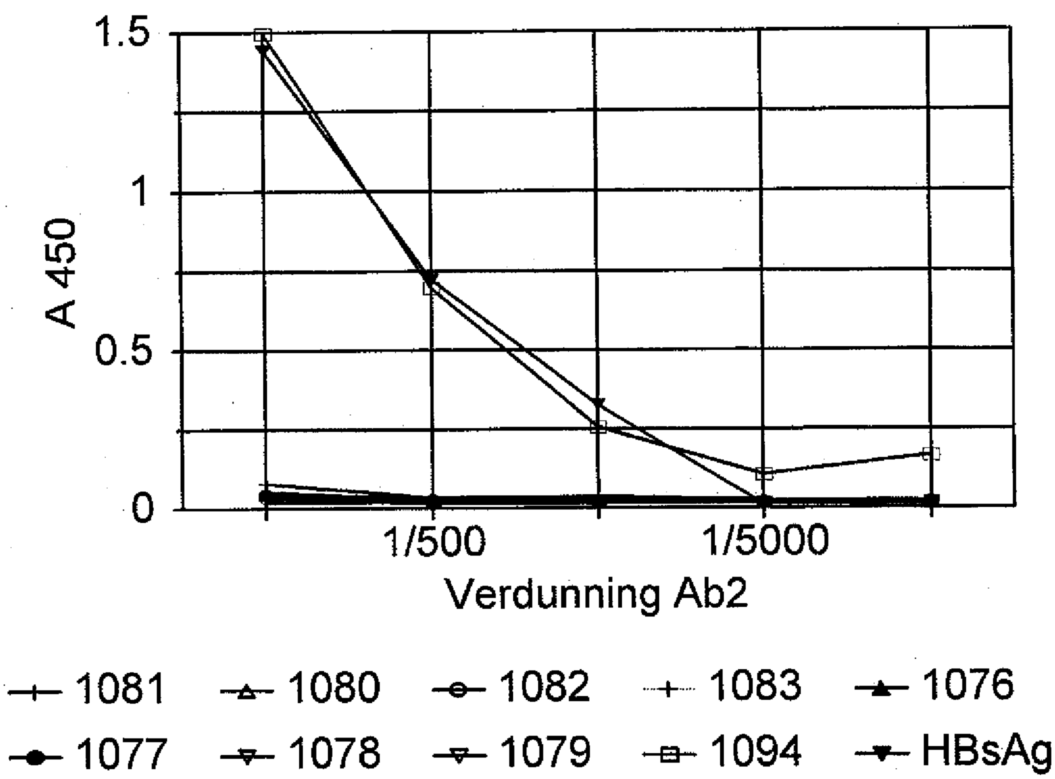

1.5
1
0.5
0
A 450
1/500
1/5000
Verdunning Ab2
1081
1080
1082
1083
1076
1077
1078
1079
1094
HBsAg

Figure 3a

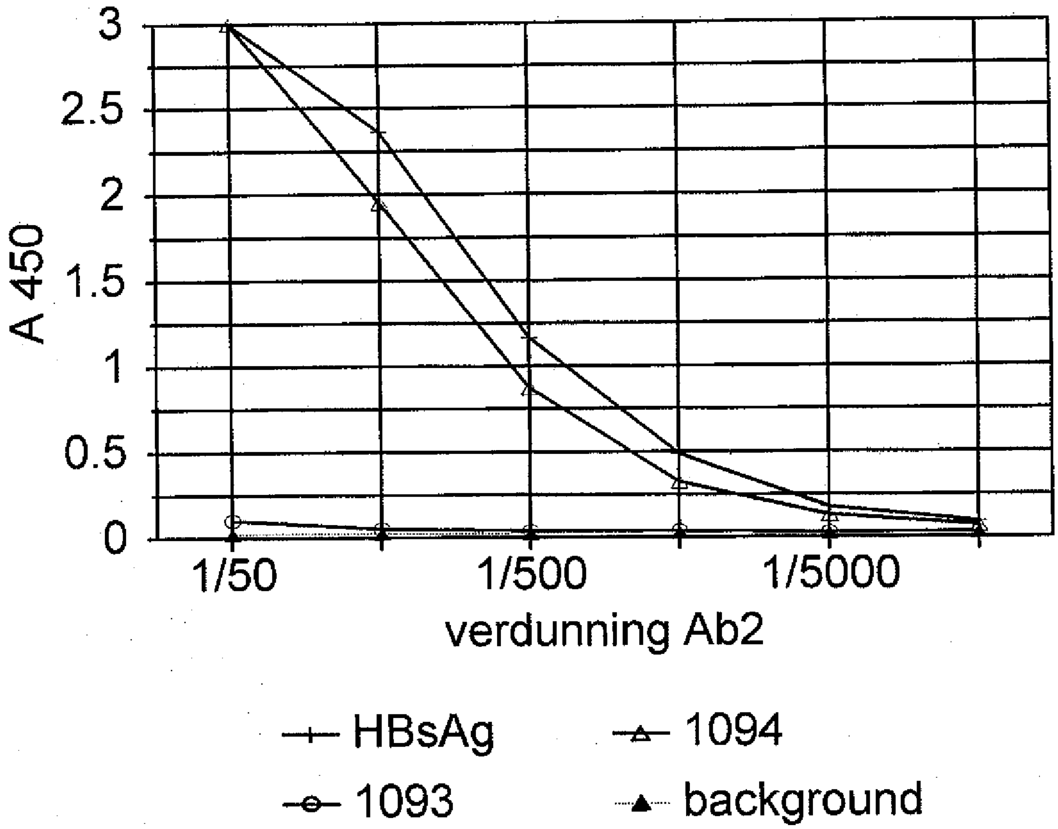

3
2.5
2
1.5
1
0.5
0
A 450
1/50
1/500
1/5000
verdunning Ab2
HBsAg
1094
1093
background

Figure 3b

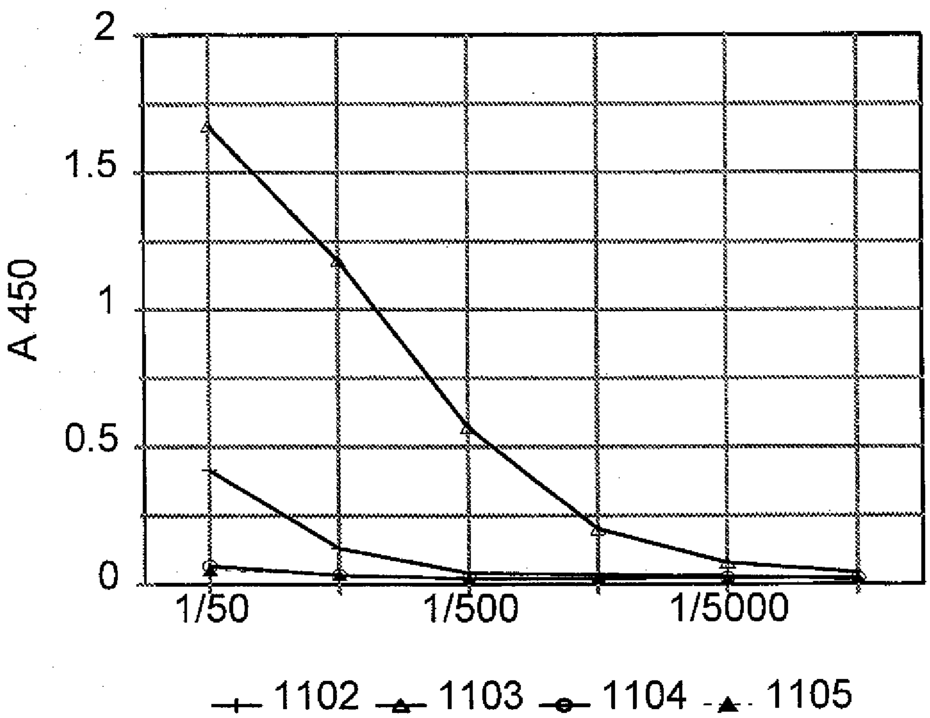
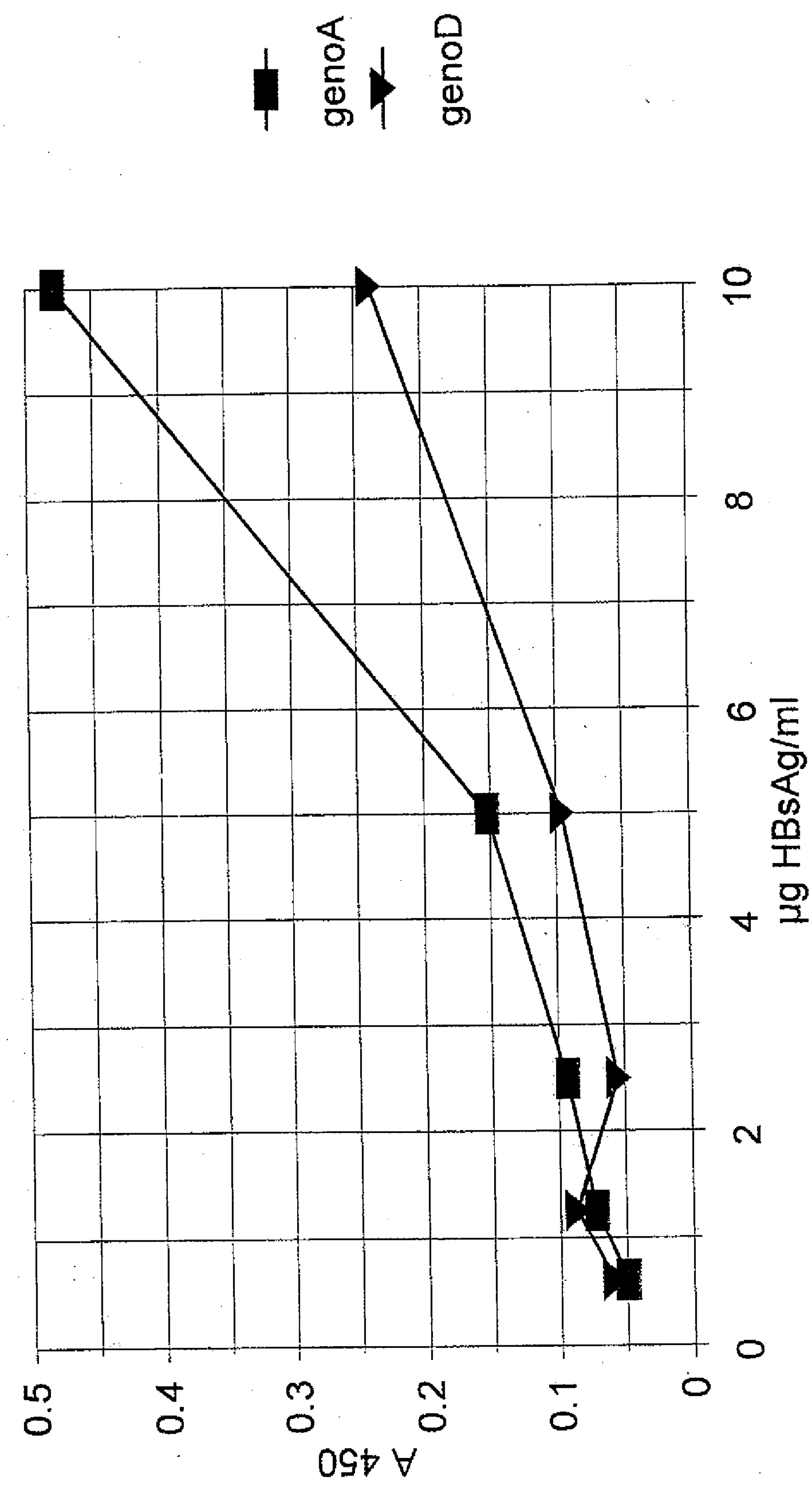


Figure 4


genoA
genoD
A 450
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
µg HBsAg/ml

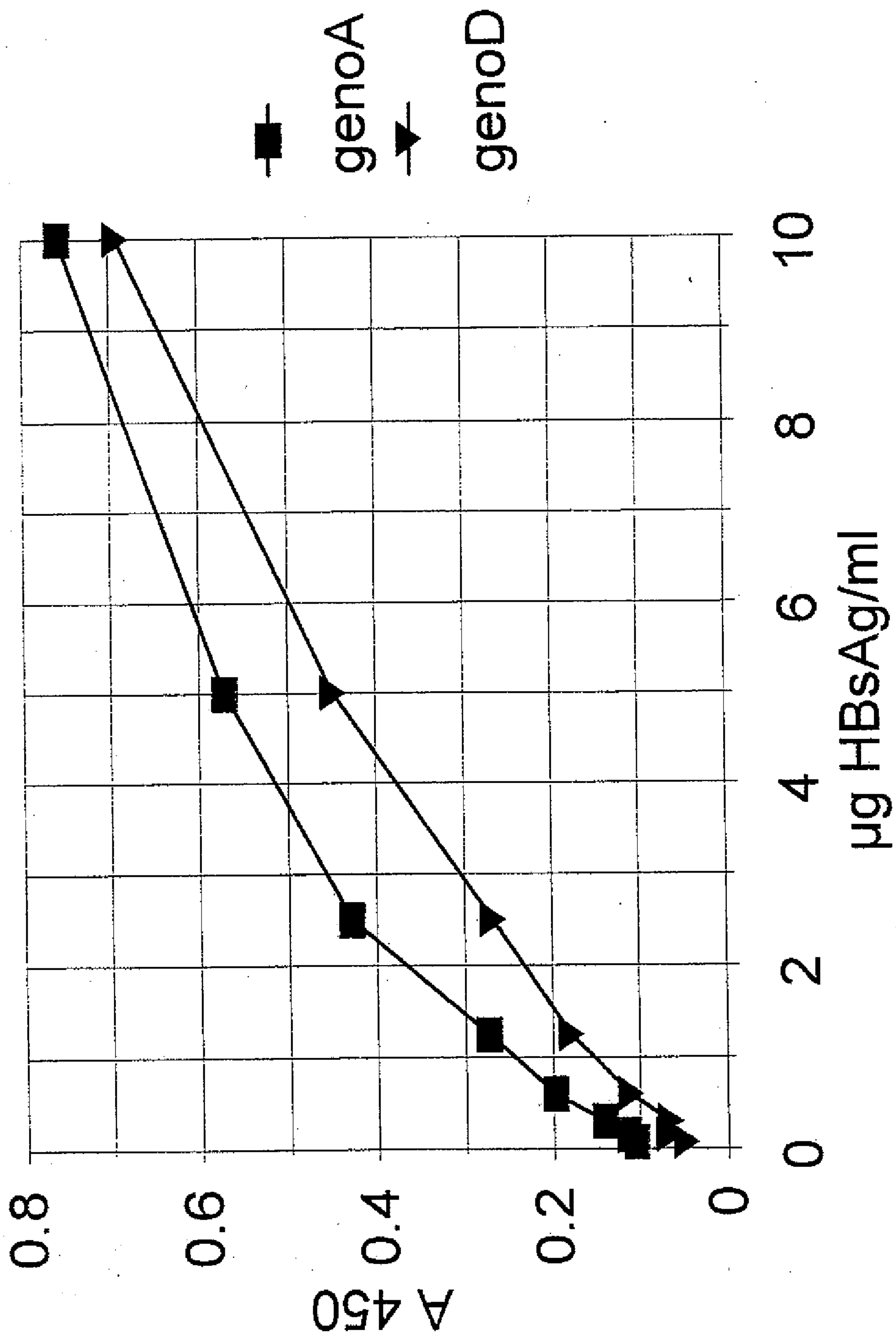


Figure 5b

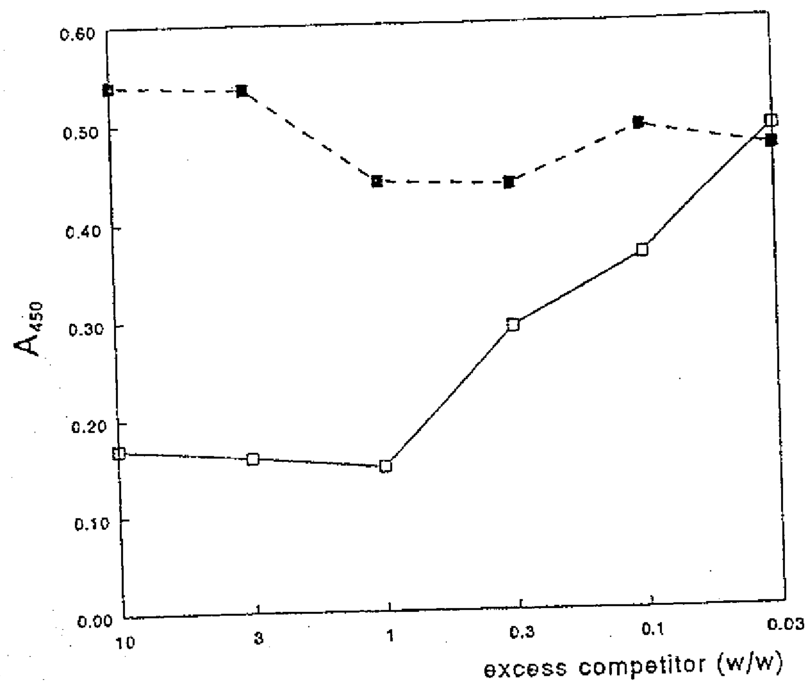

0.60
0.50
0.40
0.30
0.20
0.10
0.00
A_{450}
10
3
1
0.3
0.1
0.03
excess competitor (w/w)

Figure 6

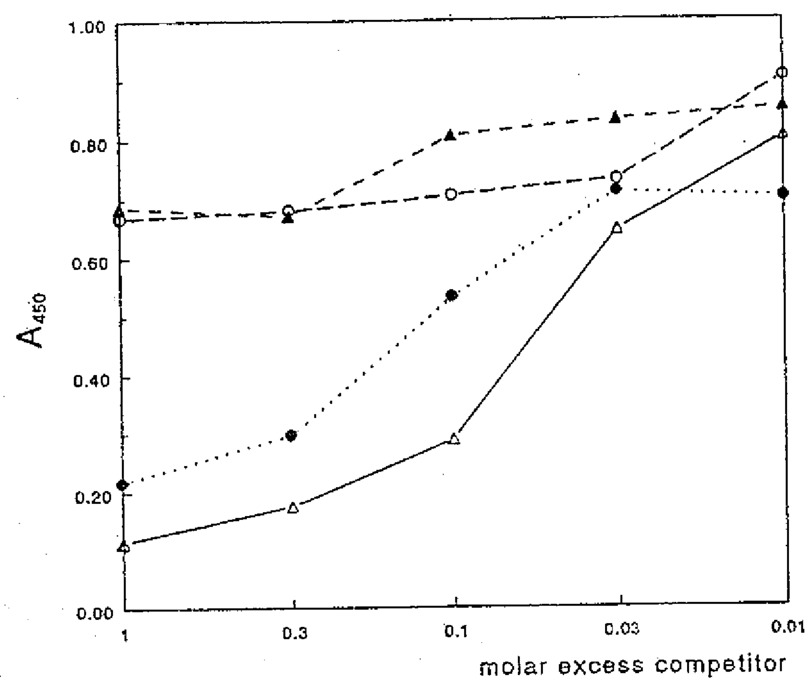

1.00
0.80
0.60
0.40
0.20
0.00
A_{450}
1
0.3
0.1
0.03
0.01
molar excess competitor

Figure 7

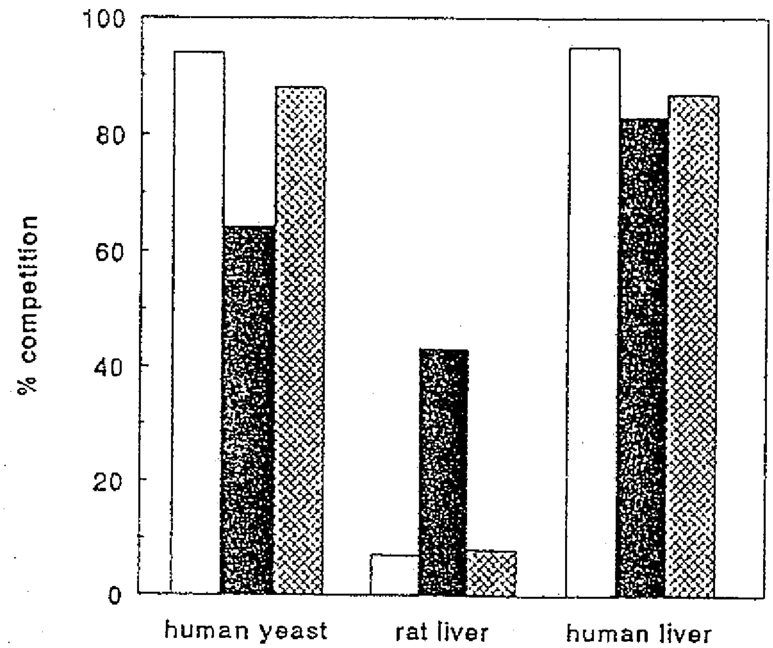

100
80
60
40
20
0
% competition
human yeast
rat liver
human liver

Figure 8

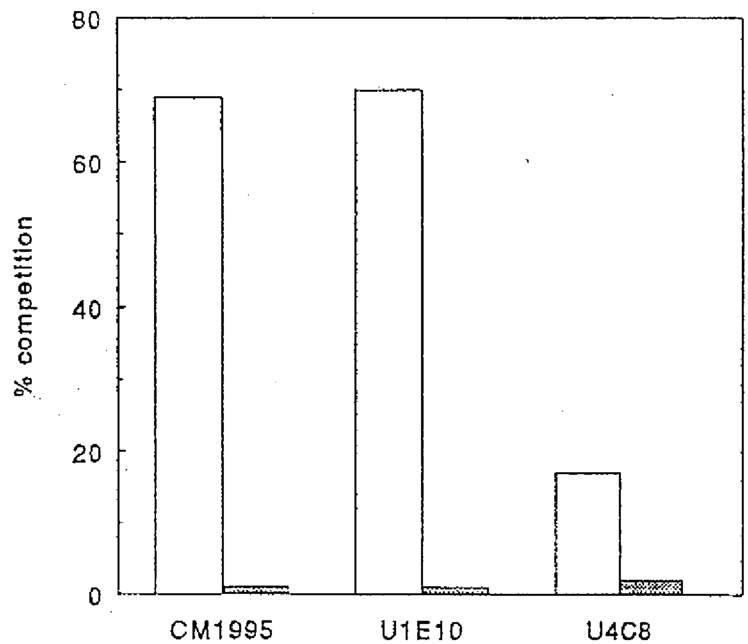


Figure 9

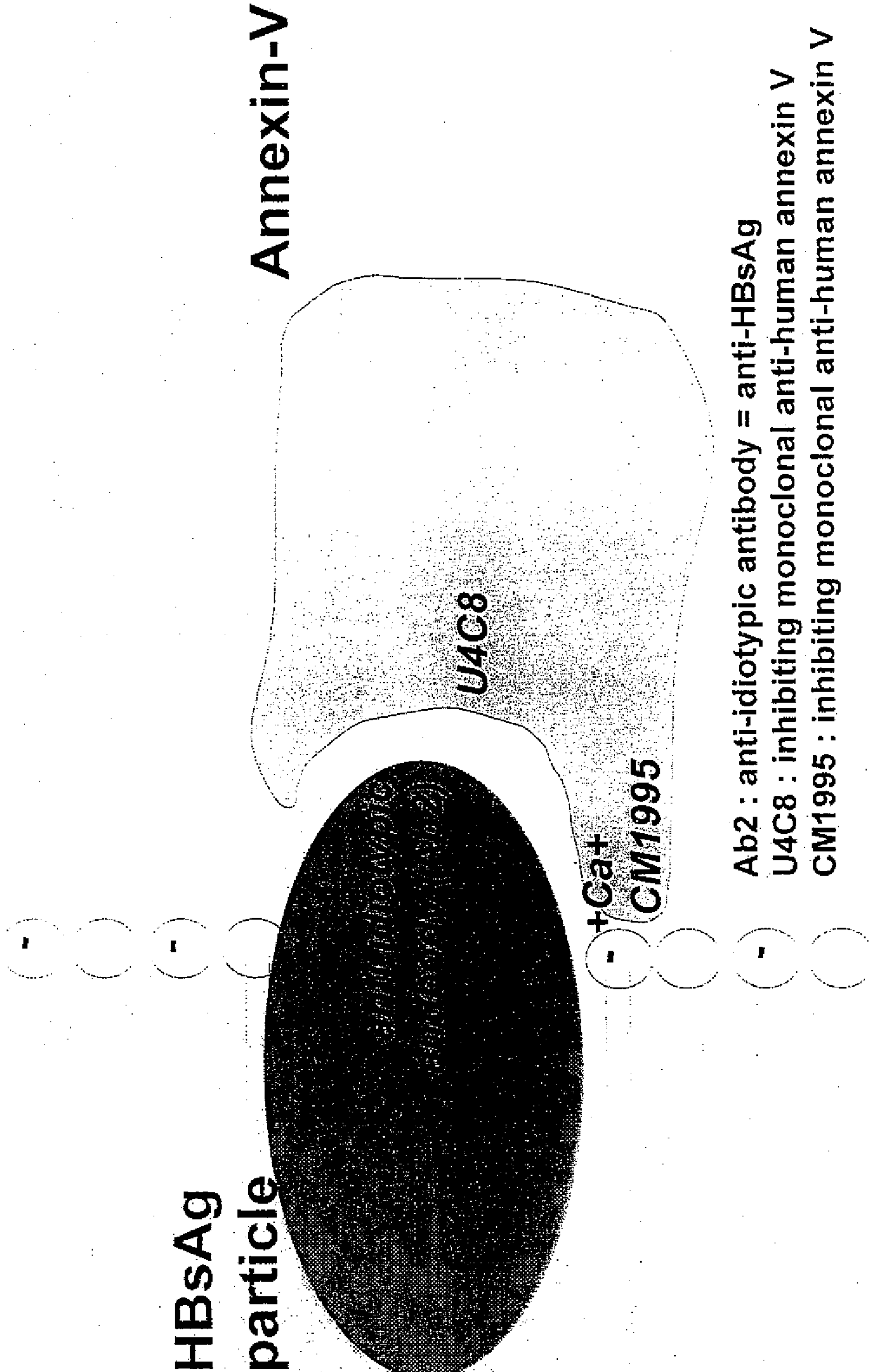

Annexin-V
U4C8
+Ca+
CM1995
HBsAg
particle
Ab2 : anti-idiotypic antibody = anti-HBsAg
U4C8 : inhibiting monoclonal anti-human annexin V
CM1995 : inhibiting monoclonal anti-human annexin V

Figure 10

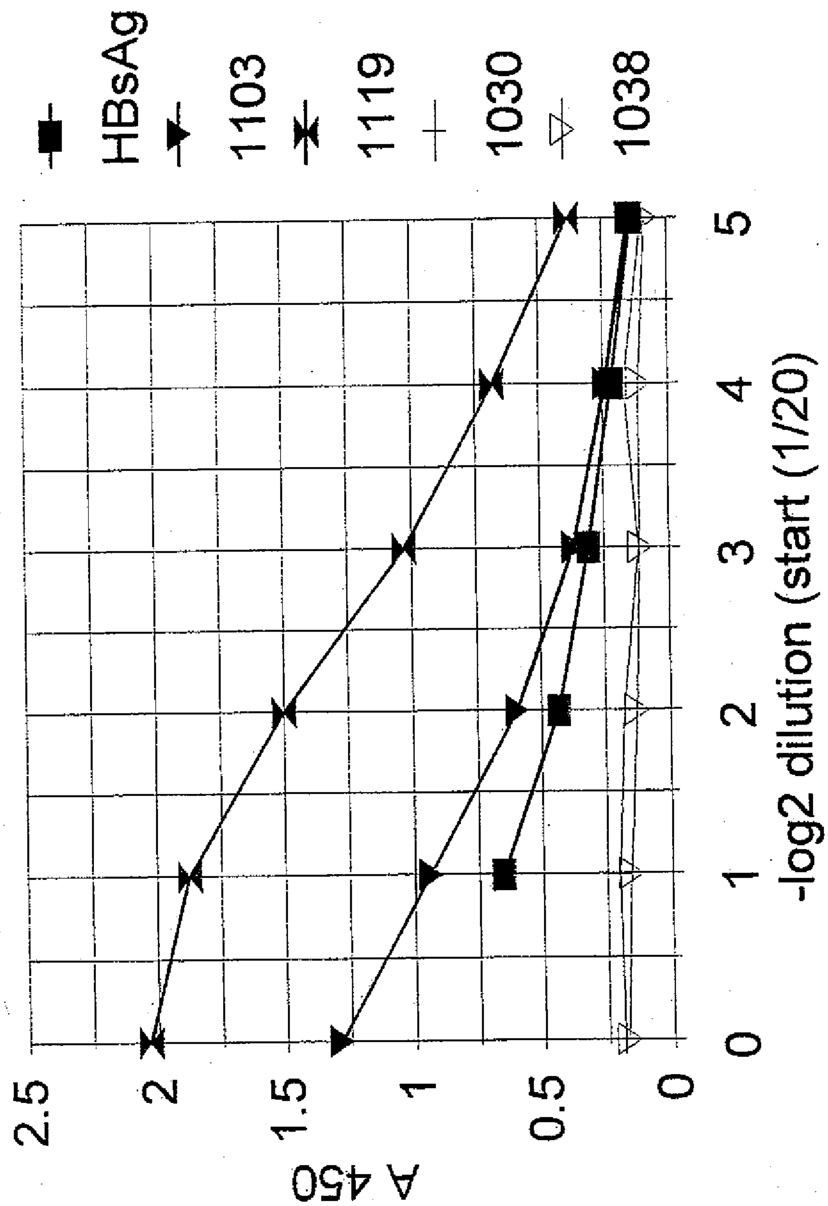

HBsAg
1103
1119
1030
1038
A 450
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
-log2 dilution (start (1/20))

Figure 11

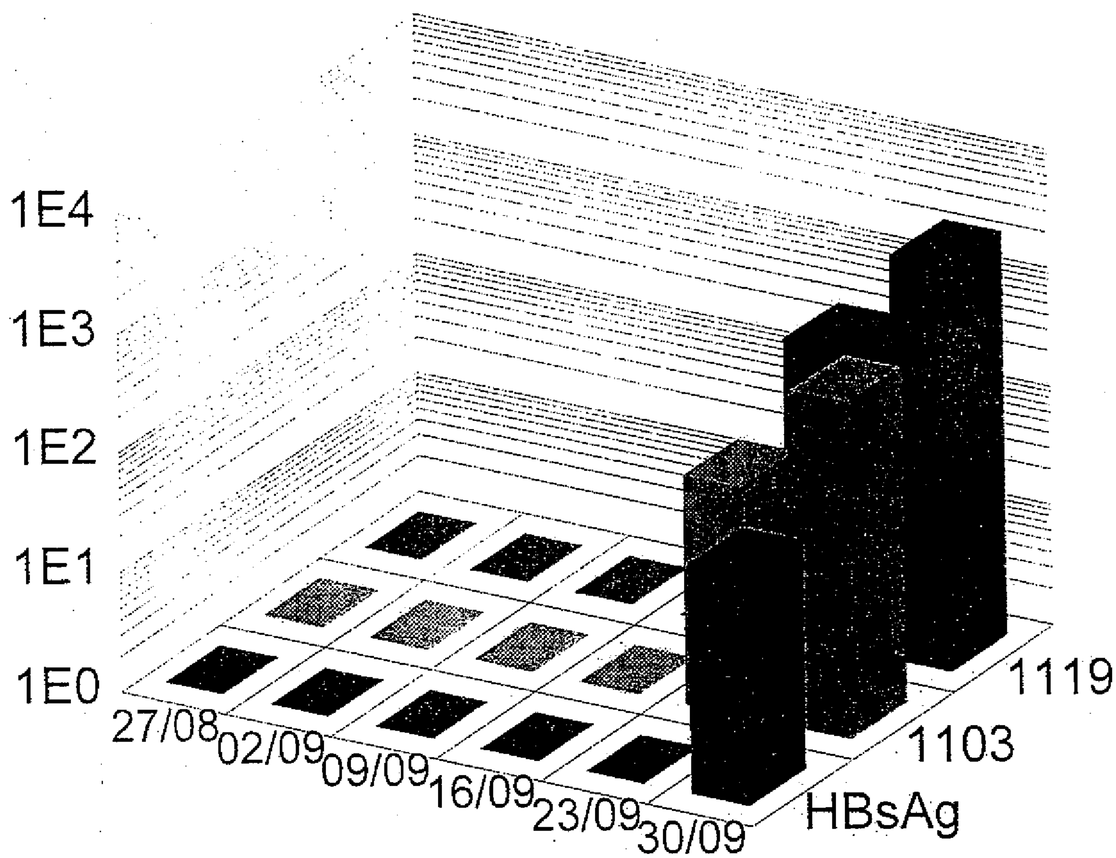

1E4
1E3
1E2
1E1
1E0
27/08
02/09
09/09
16/09
23/09
30/09
HBsAg
1103
1119

Figure 12

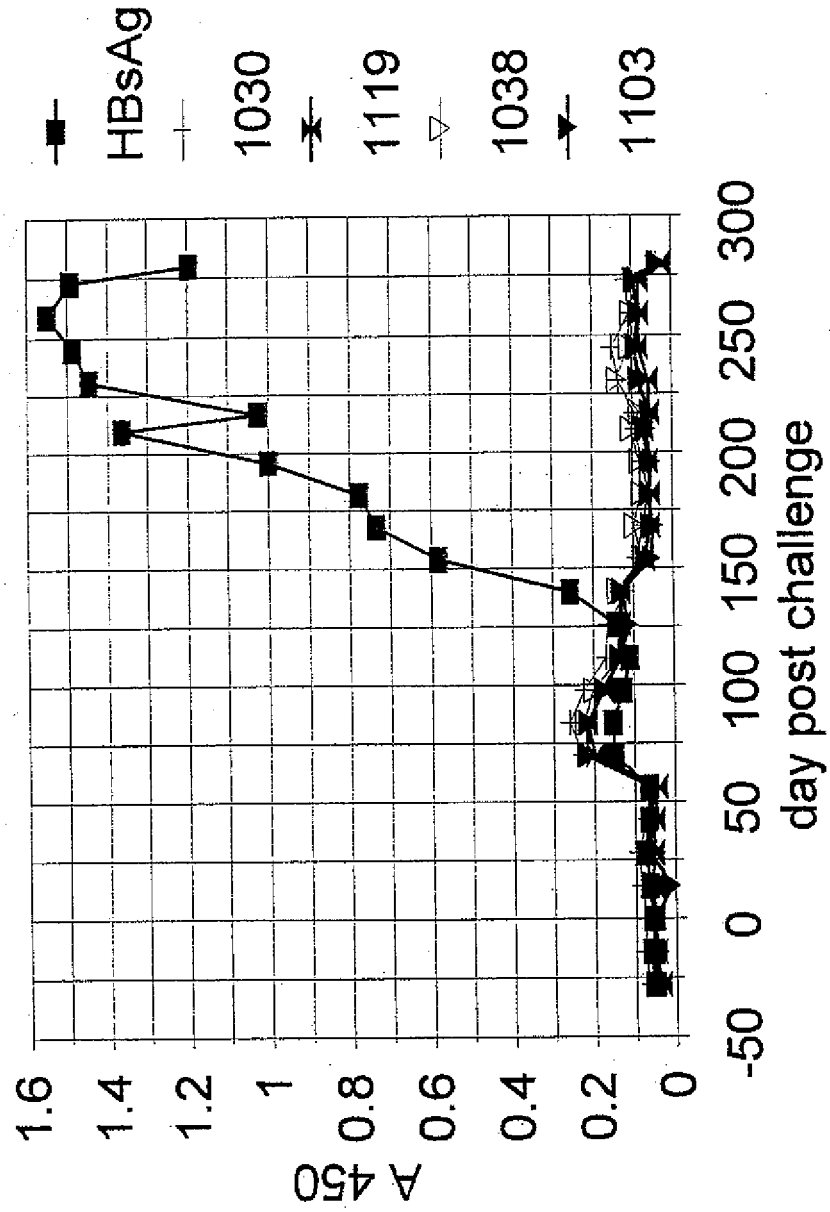

HBsAg
1030
1119
1038
1103
A 450
day post challenge
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-50
0
50
100
150
200
250
300

Figure 13

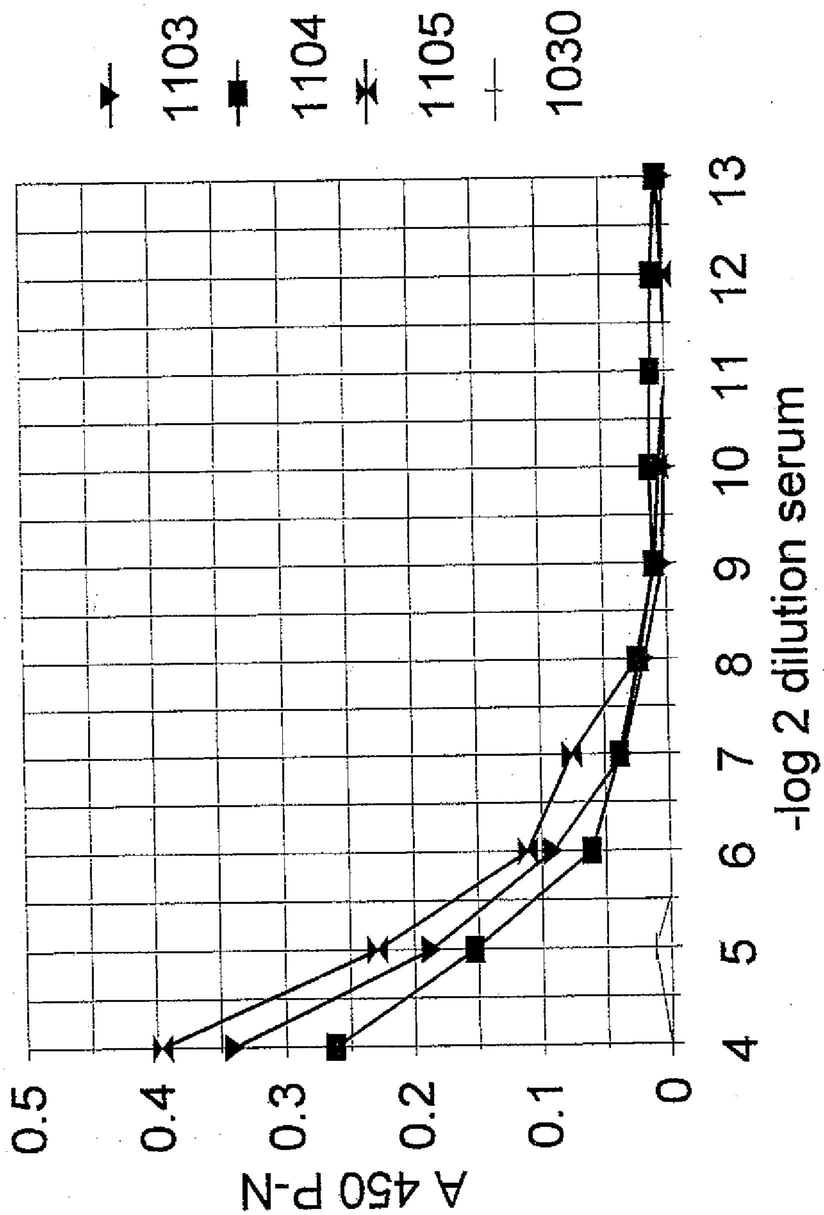

1103
1104
1105
1030
0.5
0.4
0.3
0.2
0.1
0
A 450 P-N
4
5
6
7
8
9
10
11
12
13
-log 2 dilution serum

Figure 14

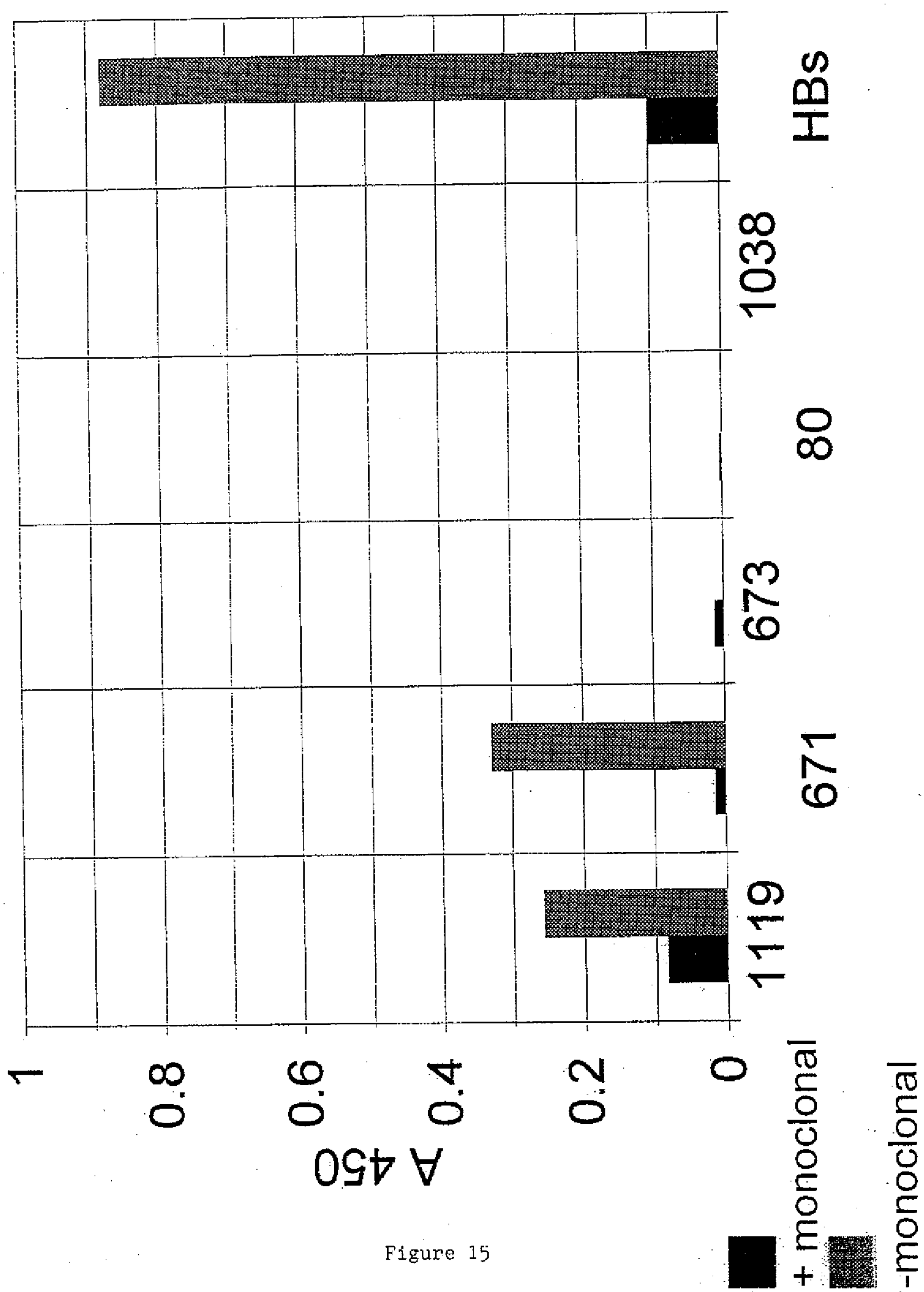

A 450
1
0.8
0.6
0.4
0.2
0
1119
671
673
80
1038
HBs
+ monoclonal
-monoclonal

Figure 15

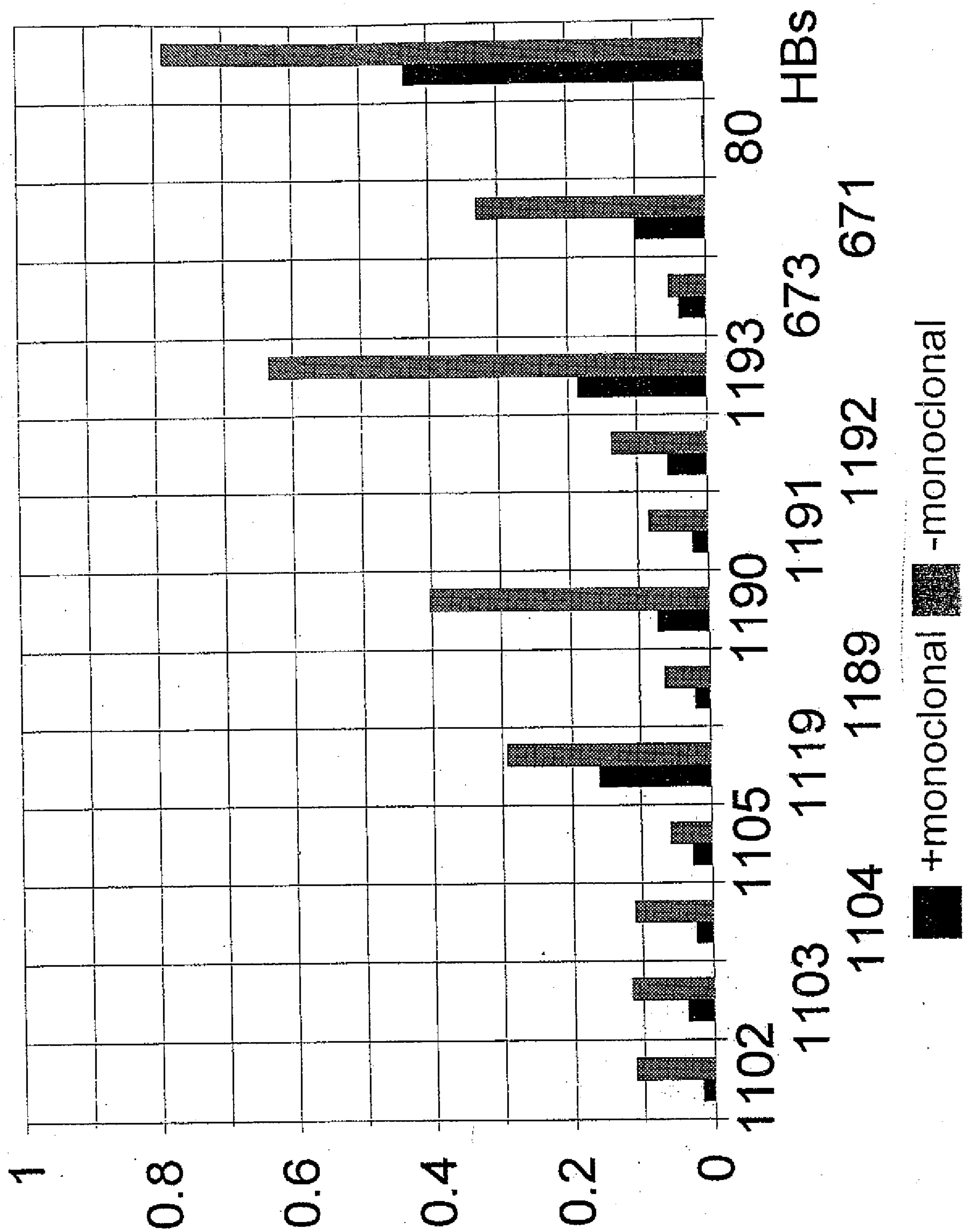

1
0.8
0.6
0.4
0.2
0
1102
1103
1104
1105
1119
1189
1190
1191
1192
1193
673
671
80
HBs
+monoclonal
-monoclonal

Figure 16

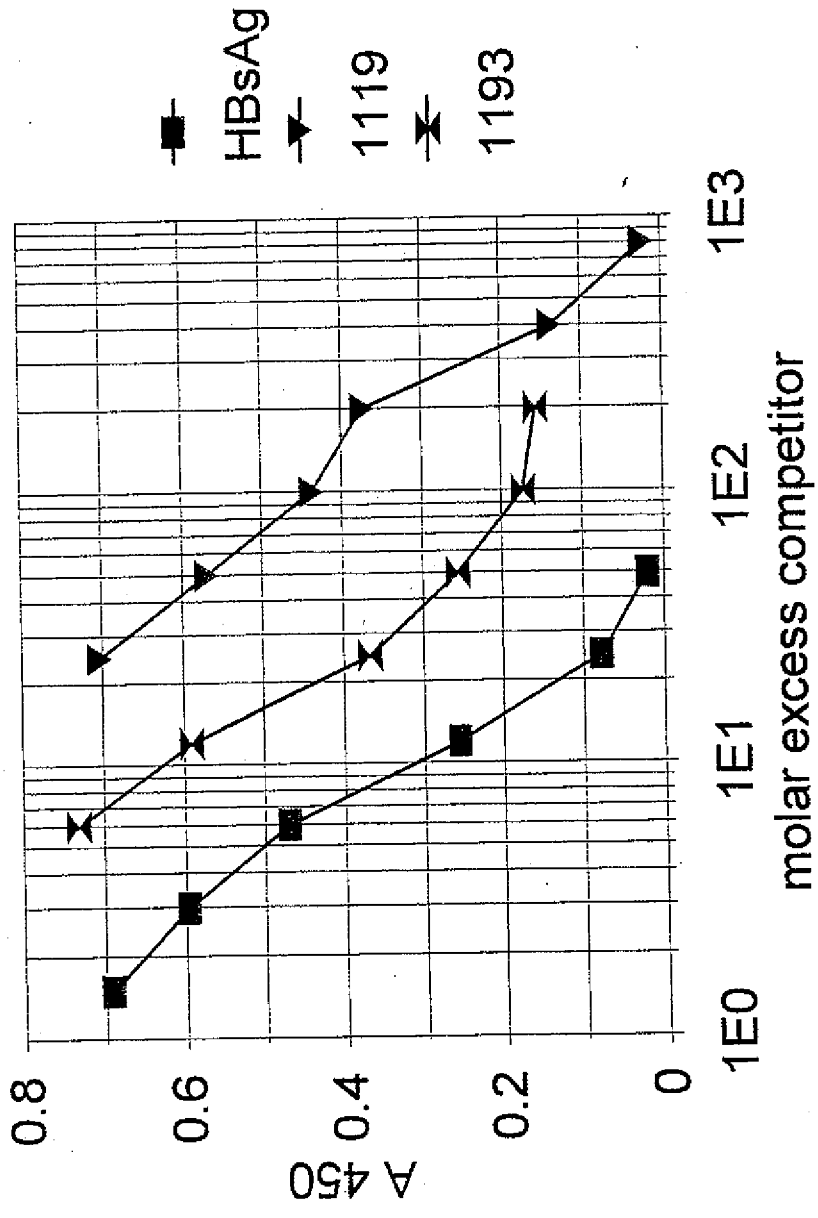
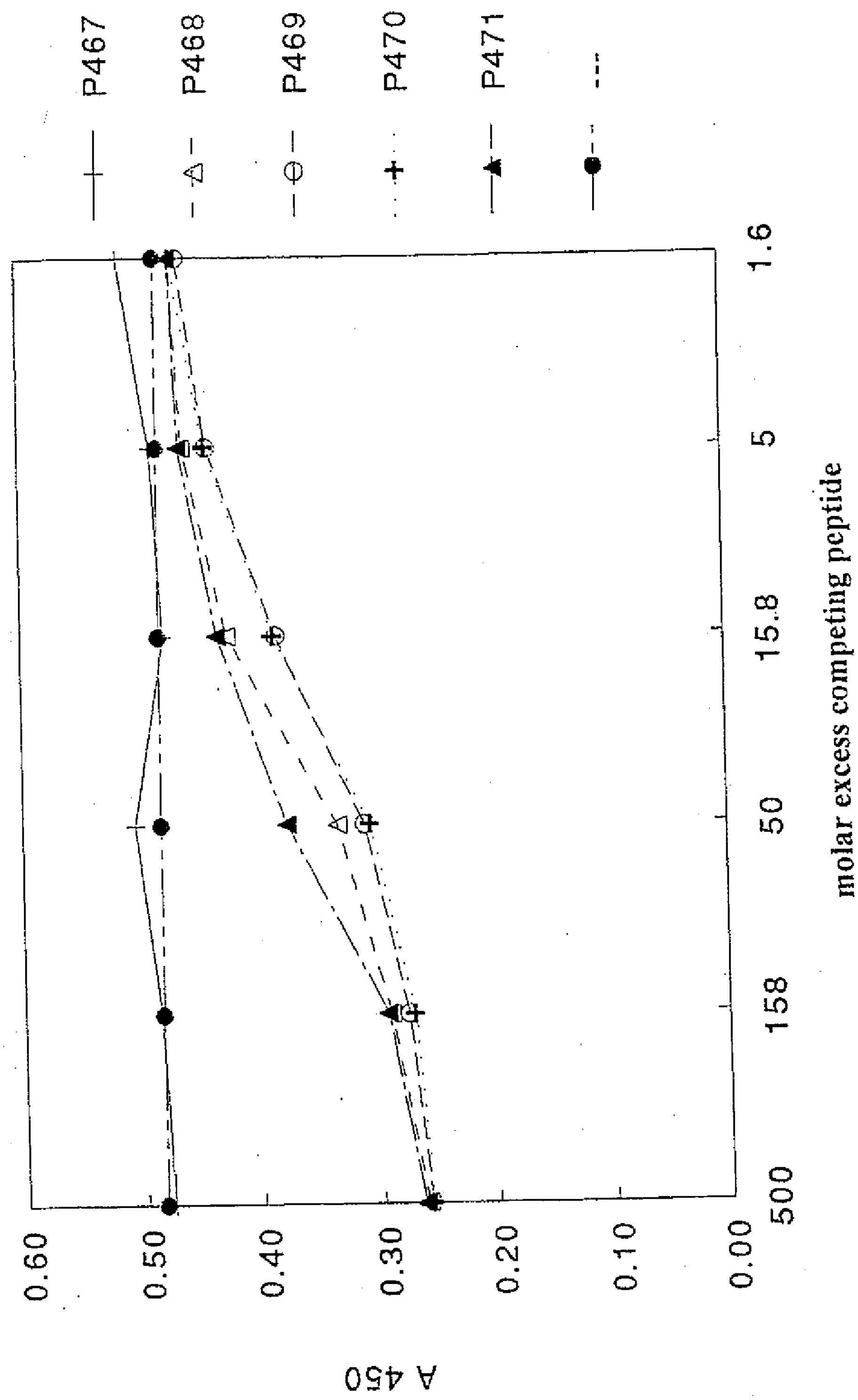

HBsAg
1119
1193
A 450
0.8
0.6
0.4
0.2
0
1E0
1E1
1E2
1E3
molar excess competitor

Figure 17


P467
P468
P469
P470
P471

0.60
0.50
0.40
0.30
0.20
0.10
0.00
A 450
500
158
50
15.8
5
1.6
molar excess competing peptide

Figure 18

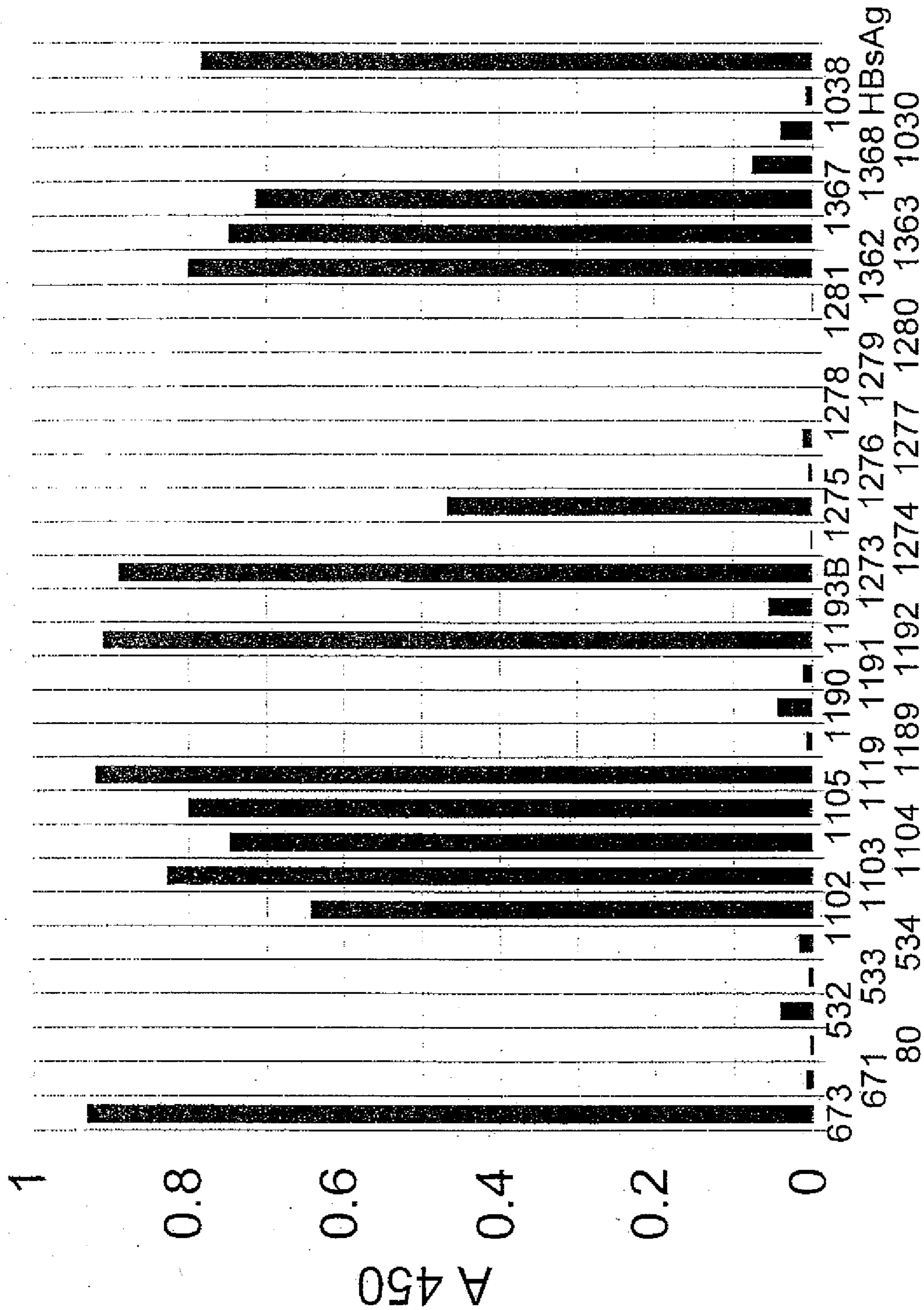

A 450
0
0.2
0.4
0.6
0.8
1
673
671
80
532
533
534
1102
1103
1104
1105
1119
1189
1190
1191
1192
1193B
1273
1274
1275
1276
1277
1278
1279
1280
1281
1362
1363
1367
1368
1030
1038
HBsAg

Figure 19

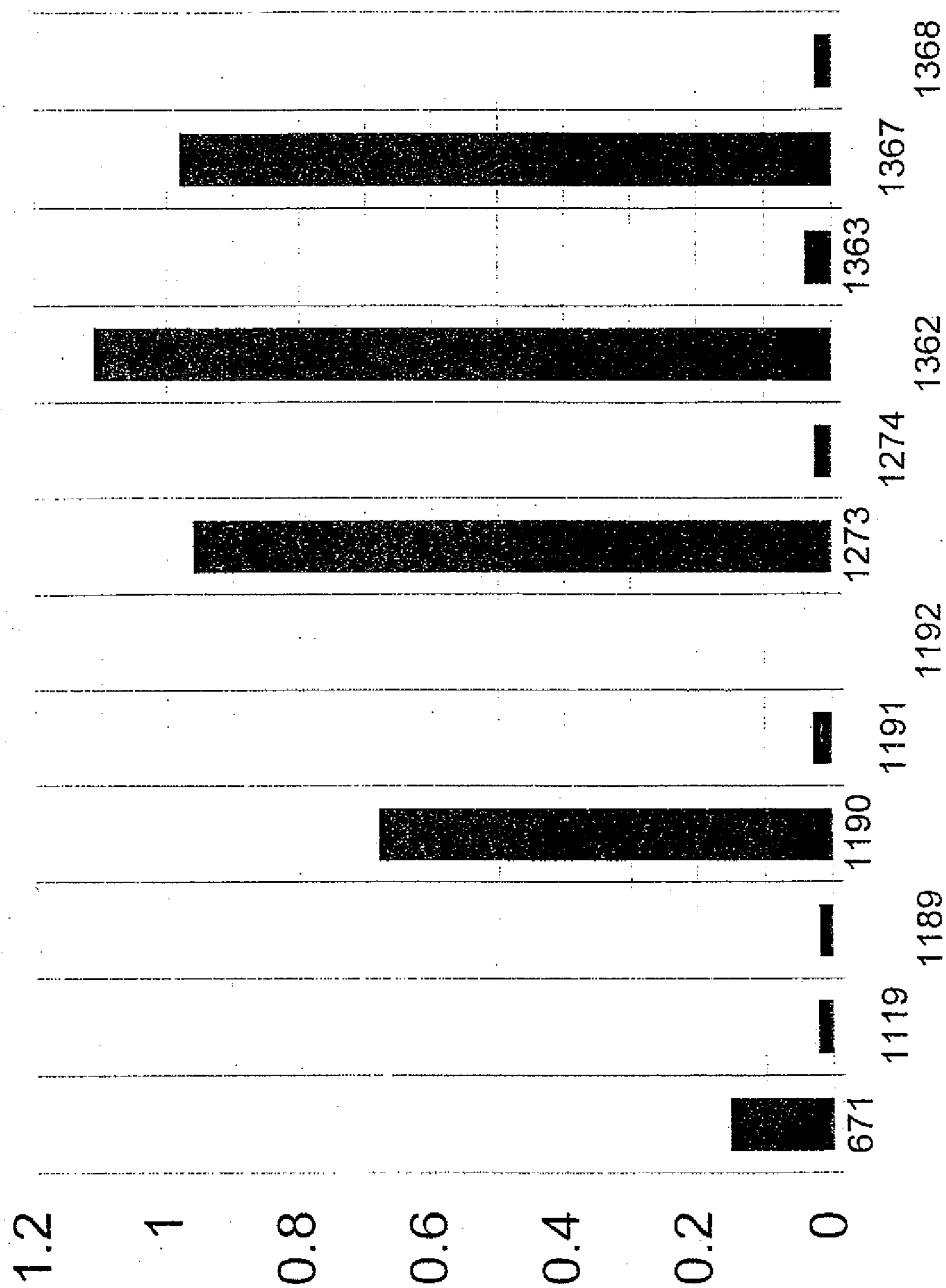

1368
1367
1363
1362
1274
1273
1192
1191
1190
1189
1119
671
1.2
1
0.8
0.6
0.4
0.2
0

Figure 20

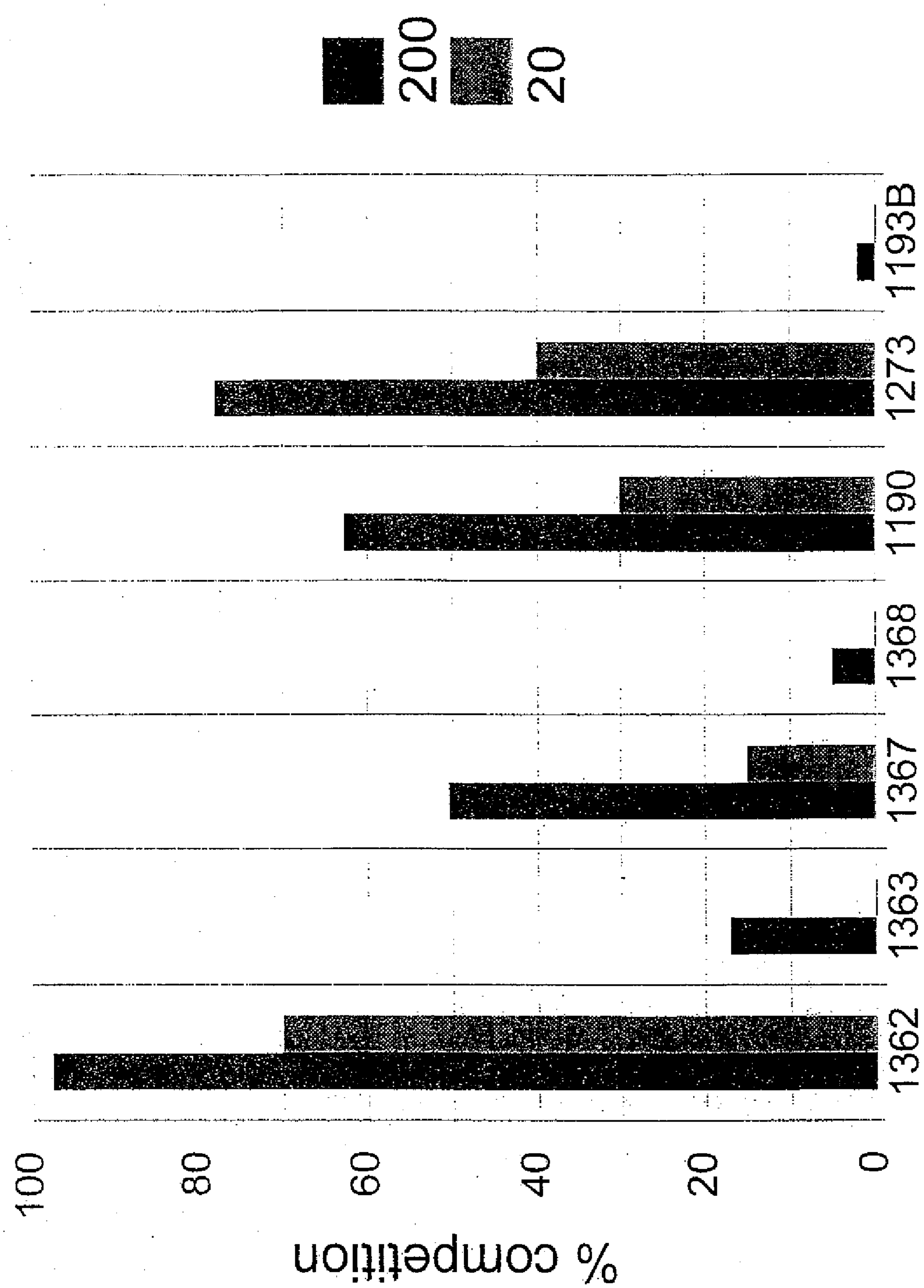

200
20
1193B
1273
1190
1368
1367
1363
1362
100
80
60
40
20
0
% competition

Figure 21

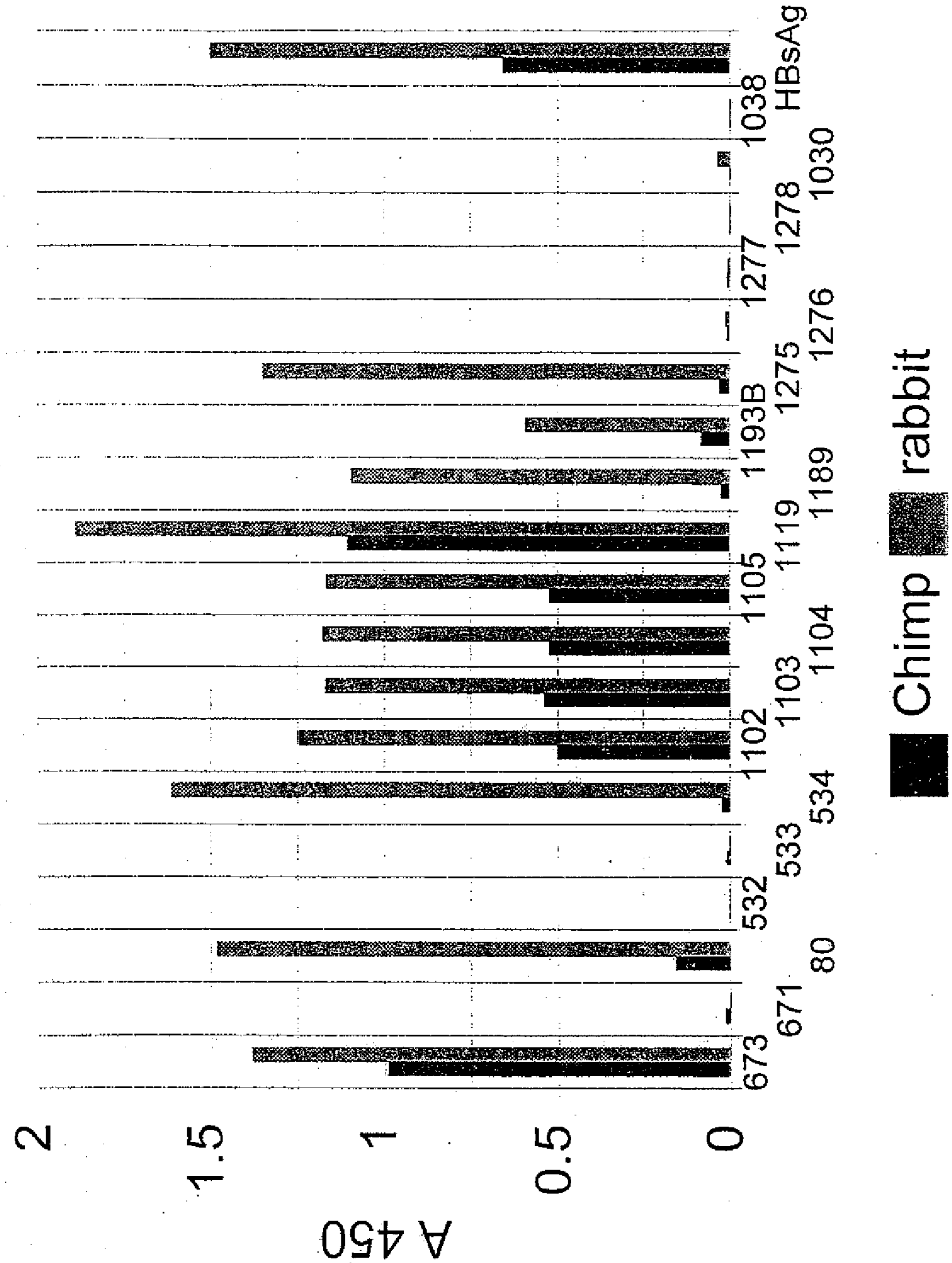

A 450
0
0.5
1
1.5
2
673
671
80
532
533
534
1102
1103
1104
1105
1119
1189
1193B
1275
1276
1277
1278
1030
1038
HBsAg
Chimp
rabbit

Figure 22

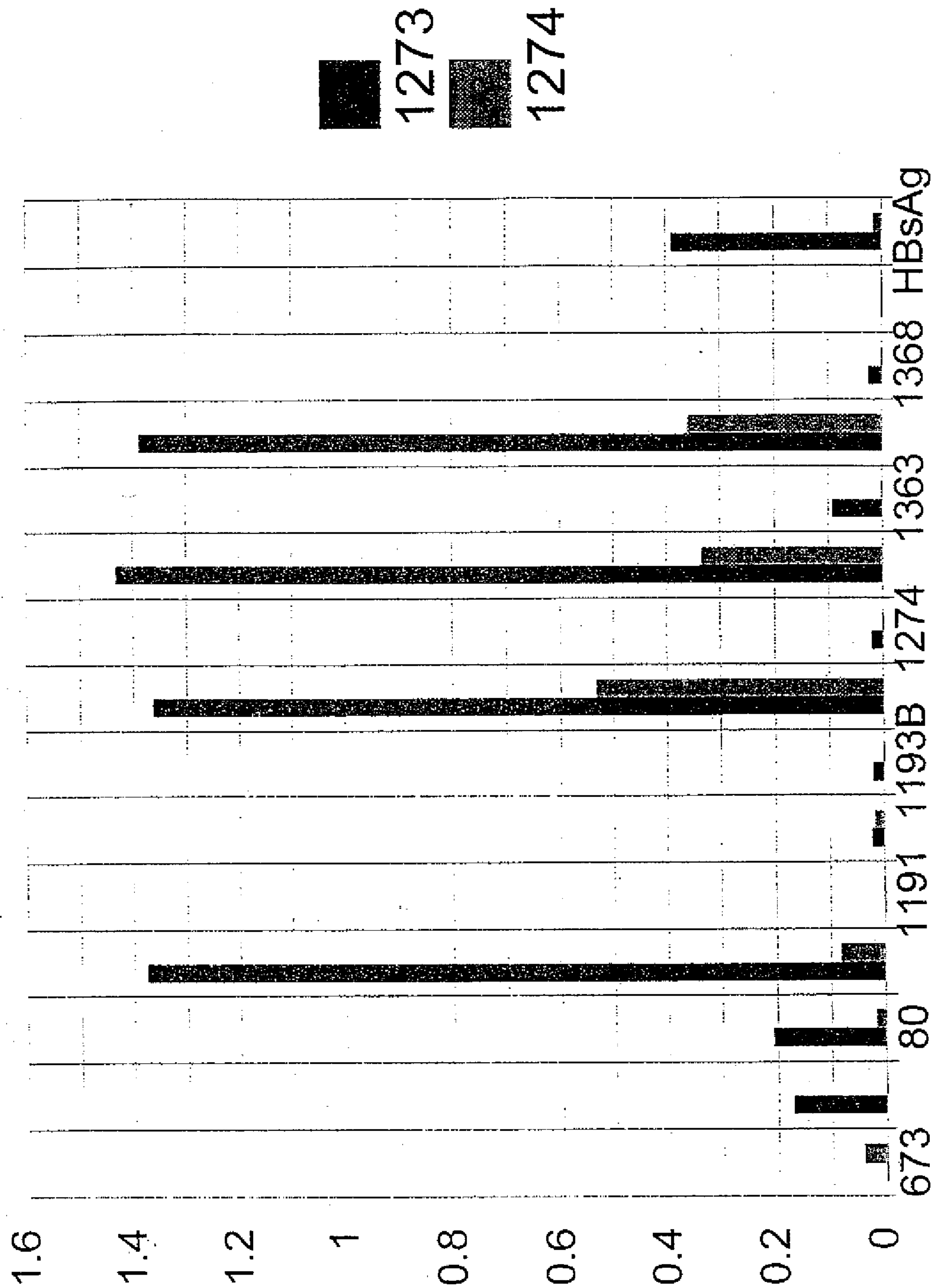

1273
1274
HBsAg
1368
1363
1274
1193B
1191
80
673
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0

Figure 23

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 97/07268

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/02 C07K16/08 A61K39/29

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 16704 A (SMITHKLINE BEECHAM BIOLOG ;LEROUX ROELS GEERT (BE); SLAOUI MOHAMED) 22 June 1995 see claims; figures 1,2; examples ---	1-10
X	EP 0 044 710 A (SCRIPPS CLINIC RES) 27 January 1982 * page 76, compounds v and w * * page 77, compound tt * see claims; examples ---	1-9
X	EP 0 421 635 A (WELLCOME FOUND) 10 April 1991 see page 9, line 32 - page 10, line 13; claims; examples ---	1-7
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
29 May 1998	12/06/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fuhr, C

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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/EP 97/07268

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 05698 A (UNITED NATIONS IND DEV ORGANIZ ;MANIVEL VENKATASAMY (IN); RAO KANU) 17 March 1994 see claims; examples ---	1-7
X	EP 0 082 789 A (BAYLOR COLLEGE MEDICINE) 29 June 1983 see claims; examples ---	1-7
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A	WO 94 01554 A (INNOGENETICS NV ;YAP SING HIEM (BE)) 20 January 1994 cited in the application see the whole document ---	1-10
X	EP 0 534 615 A (CYTEL CORP) 31 March 1993 see Seq.Id. 10 see page 10, line 39 - page 14, line 12; claims; examples ---	1-8, 10-14
P,X	WO 97 40164 A (MUREX DIAGNOSTICS CORP ;TEDDER RICHARD SETON (GB); IJAZ SAMREEN (G) 30 October 1997 see page 18, line 20 - line 31; figure 1 ---	1-4,7,10
X	DATABASE WPI Section Ch, Week 8540 Derwent Publications Ltd., London, GB; Class B04, AN 85-246196 XP002066500 -& JP 60 162 000 A (FUJISAWA PHARM CO LTD) see compound II see abstract -----	1-4,6-8, 10-14

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International Application No
PCT/EP 97/07268

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INTERNATIONAL SEARCH REPORT

Information on patent family members

In tional Application No
PCT/EP 97/07268

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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